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English translation of the International Patent Application

Improved Method for Detecting Acid Resistant Microorganisms in the Stool

The description of this invention mentions a number of published documents. The subject matter of these documents is herewith incorporated into the specification by reference.

The invention relates to a method for detecting an infection of a mammal with an acid-resistant microorganism, wherein (a) a stool sample of the mammal is incubated with (aa) a receptor under conditions permitting a complex formation of an antigen from the acid resistant microorganism with the receptor; or (ab) two different receptors under conditions permitting a complex formation of an antigen of the acid-resistant microorganism with the two receptors and wherein the receptor according to (aa) or the receptors according to (ab) specifically bind(s) an antigen which shows, at least with some mammals, a structure after passage through the intestine that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunized with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; and (b) wherein the formation of at least one antigen-receptor complex according to (a) is detected. Preferably, the acid-resistant microorganism is a bacterium, in particular *Helicobacter pylori, Helicobacter hepaticus, Campylobacter jejuni* or *Mycobacterium tuberculosis*. Moreover, the receptor(s) preferably bind(s) to (an) epitope(s) of a catalase, a metalloproteinase or urease. Furthermore, the invention relates to diagnostic and pharmaceutical compositions and test devices containing said components and packaging containing the same.

Today, there are various invasive, semi-invasive or non-invasive methods for detecting the infection of a mammalian organism with a microbial pathogen or

parasite. All invasive methods presuppose endoscopy and biopsy. If an invasive technique is used, the physical integrity of the examined subject is violated, e.g. in a biopsy. Obtaining a specimen by biopsy is time-consuming, costly and mostly puts a strain on the patient. As the infection with particular microorganisms, for instance with H. pylori, need not be distributed over the entire gastric mucosa, obtaining a specimen by biopsy at a non-infected site may deliver a false-negative result. Another disadvantage of all invasive methods is that all examination results are influenced by an earlier treatment with proton-pump inhibitors, bismuth or antibiotics.

Semi-invasive or non-invasive diagnostic methods note changes in parameters which may be measured without interfering in the organism. For this purpose preferably samples of body fluids and excretions, such as serum, breath, urine, saliva, sweat or stool are taken and analysed. With direct methods the presence of the pathogen or parasite, its components or their degradation products is detected by electron microscopy, optical characterisation, mass spectrometry, measurement of the radioactive degradation products or specific enzymatic reactions. However, these methods often require expensive and sophisticated instruments (e.g. the breath test). By contrast, indirect methods are used for detecting reactions of the host organism to the pathogen or the parasite, for instance the presence of antibodies against antigens of the pathogen in the serum or the saliva of the host. Since interfering in the organism using invasive techniques strains the organism in most cases and also requires expensive and sophisticated instruments and involves health hazards, non-invasive techniques are the methods of choice since it is comparatively simple to take samples of the above-mentioned body fluids and excretions. Furthermore, since not every host reacts in the same way to a particular pathogen or parasite, and the host's reaction is delayed and may persist even after the pathogen or parasite has been removed from the organism, direct methods should always be preferred. Hence, ideally, a diagnosis is made by means of the non-invasive, direct detection of the pathogen or parasite in body fluids or excretions. Contrary to indirect methods, this allows the current infection status to be determined.
Moreover, a diagnostic method should also be optimised with regard to other aspects: high reproducibility, sensitivity and specificity, guaranteed availability and constant quality of the materials to be used, low costs for producing and carrying out the method and simple application independent of expensive and sophisticated instruments are parameters to be taken into consideration.
For the above-mentioned reasons, in medical diagnostics increasing use is made of methods based on the high selectivity and binding affinity of particular classes of substances (e.g. antibodies, receptors, lectins, aptamers) for molecular structures which can be selected in such a way that they are highly specific for the

corresponding substance to be analysed. It was mainly the possibility of immobilizing these substances on the surface of solids as well as the coupling of radioactive nuclides, of enzymes triggering colour reactions with suitable substrates or of coloured particles with a highly specific binding affinity (e.g. in ELISA = enzyme-linked immunosorbent assay) that led to the development of inexpensive, simple and less timely methods for detecting substances that are naturally-occurring in the body or foreign to body.

In the initial phases of the development of these detection methods exclusively polyclonal antibodies were used. They, however, have several disadvantages well known to the person skilled in the art, chief among these are limited availability and often cross-reactivity. The development of methods for preparing monoclonal antibodies (Köhler & Milstein (1975)), the advances in the isolation of receptors and their directed expression in cellular host systems, the development of lectins with high affinity to particular carbohydrates and the discovery that single-stranded nucleic acid molecules (aptamers) are able to specifically bind molecular structures, allowed the majority of these disadvantages to be eliminated. Today, the specificity and sensitivity of detection methods can be optimised with comparatively simple methods.

Due to the high specificity, such methods are particularly suitable for detecting individual, defined substances such as haptens, peptides or proteins, provided the structural element that has been recognised is constant within the specimen population to be examined and specific to the substance to be detected. Moreover, they are well suited for measurements in body fluids and, thus, are an obvious option for the direct detection of pathogens in this specimen matrix. Accordingly, the prior art describes methods for diagnosing e.g. *Entamoeba histolytica* (Hague (1993), J. Infect. Dis. 167: 247-9), enterohemorrhagic *Escherichia coli* (EHEC, Park (1996), J. Clin. Microbiol. 34: 988-990), *Vibrio cholerae* (Hasan (1954), FEMS Microbiol. Lett. 120: 143-148), Toro virus-like particles (Koopmans (1993) J. Clin. Microbiol. 31: 2738-2744) or *Taenia saginata* (Machnicka (1996), Appl. Parasitol. 37: 106-110) from stool.
The feature the above-described pathogens have in common is that they are viable and reproducible in the intestine of the host, in all cases humans. Hence, they have mechanisms allowing them to survive and propagate in the presence of the degradation and digestion systems active in the intestine. Thus, a large number of intact or almost intact pathogens or parasites are likely to be passed with the stool. As a rule, it is easy to detect them in the stool or in prepared stool samples by means

of detection reagents, for instance antibodies that recognise the intact pathogens or parasites.

There is, however, a number of pathogens or parasites that, on the one hand, may be present in the stool due to the relations of the affected tissue (e.g. lungs, stomach, pancreas, duodenum, liver) to the gastrointestinal tract and that, on the other hand, are not viable and/or reproducible in the intestine itself. These pathogens and parasites include, for instance, *Helicobacter pylori (H. pylori)* and *Helicobacter hepatis*, *Mycobacterium tuberculosis* and other mycobacteria, *Chlamydia pneumoniae, Legionella pneumophilae, Pneumocystis carinii* and others. Some of these pathogens can be detected, for example, in the sputum. However, for example, the detection of *Mycobacterium tuberculosis* in the sputum is possible within a short period of time only, i.e. once a cavern containing the pathogen has opened. Moreover, detection is rendered more difficult due to the fact that it is not always possible to obtain a sputum sample of the subject to be examined. This applies, for instance, to infants, confused patients or animals. Other pathogens, such as *Legionella pneumophilae* can be detected specifically by means of antigens which get into the urine via the kidneys. Yet, this is only possible if the amount present in the urine is sufficient for the detection. Detection in the stool would be a good alternative. In these organisms, however, passage through the intestine is combined with a strong attack by the digestion and degradation mechanisms of the intestinal flora. In this case, molecular structures which are specific to the pathogen observed can be destroyed or their concentration can be greatly reduced.

With other acid-resistant bacteria too, the degradation of pathogens in the intestine has turned out to be a problem for reliable detection in stool samples. The number of germs in the stomach of an infected patient is small compared to the number of other bacteria settling in the intestine. Furthermore, germs and germ fragments have to pass a long way through the intestine, which is rich in proteases, after leaving the stomach. Due to these circumstances, only small amounts of intact proteins can be found in the stool. It can, however, not be assumed that always the same fragments of specific proteins pass the intestinal tract undamaged. Another consequence of this is that the combination of two epitopes on one antigen, which is necessary for ELISA, is no longer necessarily like the one occurring in the native protein and epitopes located closely to each other are most likely to show a positive result in a detection method requiring two epitopes on the same molecule. Ideally, only one epitope on the same molecule is needed for detection. In addition, the distribution of antigens detected in the stool of infected patients suggests individual features in the processing of the antigens during passage through the intestine. A first approach to

reduce this problem has been provided by the disclosure of EP-A 0 806 667. In this application it was shown that polyclonal antibodies could be induced with the lysate of a particular *H. pylori* strain. These antibodies recognise a greater variability of strains from different geographical regions. However, this application does not indicate which antigens are recognised by the serum. In view of the fact that immune sera may vary in spite of all standardisation efforts, the method developed in the above-mentioned application must be regarded as suboptimal for broad application. In addition, it is necessary to keep immunizing new animals in order to provide polyclonal sera. The corresponding methods are both time-consuming and costly.

Ideally, a single or a limited number of reagent(s) specific to this pathogenic organism/parasite should enable the reliable detection of the infection of an acid-resistant pathogenic organism/parasite as broadened above. Such a possibility would, above all, reduce the costs of corresponding detection methods considerably. Hence, the technical problem underlying the present invention was to provide a corresponding detection method or corresponding reagents.

This technical problem has been solved by providing the embodiments characterised in the claims.

Thus, the invention relates to a method for detecting an infection of a mammal with an acid-resistant microorganism, wherein (a) a stool sample of the mammal is incubated with (aa) a receptor under conditions permitting the formation of a complex of an antigen from the acid-resistant microorganism with the receptor; or (ab) two different receptors under conditions permitting the formation of a complex of an antigen from the acid-resistant microorganism with the two receptors, and wherein the receptor according to (aa) or the receptors according to (ab) specifically bind(s) to an antigen which shows, at least with some mammals, a structure after passage through the intestine that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunized with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; and wherein (b) the formation of at least one antigen-receptor complex according to (a) is detected.

Within the meaning of the present invention, the term "acid-resistant microorganism" encompasses any microorganism which, due to its properties/mechanisms of adapting to the host, withstands the physical and chemical influence of the digestive tract with the effect that it can be detected by a preferably immunological test or by the use of aptamers. Examples of such acid-resistant microorganisms are

Helicobacter pylori, Helicobacter hepaticum, Mycobacterium tuberculosis, Mycobacterium pseudotuberculosis and *Mycobacterium cansassii*.

The term "stool sample of the mammal" as used in the present invention means any stool sample that can be used for the detection method of the invention. In particular, it includes stool samples which have been prepared for diagnostic tests according to methods basically known. Preparation may be carried out, for instance, according to RIDASCREEN® Entamoeba enzyme immunoassay (R-Biopharm GmbH, Darmstadt).

The person skilled in the art may readily adjust "conditions permitting complex formation"; cf. also Harlow and Lane, ibid. These conditions are, for example, physiological conditions.
The term "shows [...] a structure after passage through the intestine that corresponds to the native structure", as used in the present invention, means that the epitope of an antigen is recognised after passage through the intestine by a receptor, e.g. a monoclonal antibody, derivative or fragment thereof or the aptamer which has been obtained against the same antigen/epitope that has not passed the intestine or which is bound thereto. In other words, the epitope/antigen that is specifically bound by the above, has passed the intestine intact or essentially intact as regards its structure and has not been degraded. A source for the native structure of the epitope/antigen may, for instance, be a bacterial extract that was disrupted by means of a French press and further purified according to standard methods (cf., for instance, Sambrook et al., "Molecular Cloning, A Laboratory Manual", 2nd edition, 1989, CSH Press, Cold Spring Harbor USA) or a bacterial lysate further purified according to standard methods (e.g. Sambrook et al., ibid.).

According to the invention, the term "shows [...] a structure after passage through the intestine that corresponds to the structure against which a mammal produces antibodies after being infected or immunized with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide" means that the epitope recognised by the receptor corresponds to an epitope which is presented by the immune system of a mammal, preferably a human. The mechanisms of antigen presentation as well as the mechanisms leading to the processing of antigens and the variety of antibodies resulting therefrom have been known in the prior art and have been described, for instance, in Janeway and Travers, Immunologie, 2nd edition 1997, Spektrum Akademischer Verlag GmbH, Heidelberg. These epitopes may differ from native epitopes. The contact of the mammal with the microorganism or proteins or fragments or the synthetic peptides

can be brought about by natural infection (except for synthetic peptides) or by immunization. For immunization, also extracts, lysates, synthetic peptides, etc. of the microorganism/protein can be used. Suitable immunization methods have been known in the prior art and have been described, for instance, in Harlow and Lane, ibid. Suitable antibodies may also be obtained, for example, by immunization and/or screening for surrogates such as synthetic peptides, recombinantly produced proteins, extracts, lysates or partially digested proteins.

"Synthetic peptides" comprise peptides having at least one epitope of the native antigen or the antigen which has passed through the intestine. The peptides can have the same primary structure as the antigen or fragments thereof. However, they can also have a different primary structure (primary amino acid sequence, for instance conservative exchanges).
The term "specifically binds ", as used herein, means that the receptor shows no or essentially no reactivity with other epitopes in samples of non-infected mammals. Usually the receptor only binds to the epitope of an antigen that is present in the stool sample.

According to this embodiment of the invention, a prepared stool sample can be bound, for instance, to a solid phase and the infecting agent can be detected with the labelled receptor. If the antigen which is present after having passed the intestine is (still) present in (homo-) dimeric or multimeric form, the same receptor can be used both as a catcher and as a detector.

In addition, it is of importance for the method of the invention that successful detection requires only one epitope of an antigenic protein to be detectable after passage through the intestine in an essentially consistent manner. This epitope can occur several times on a homodimer or -multimer. The likelihood to find this epitope in detectable form is, however, significantly higher than is the case for a detection test having to rely on more than one epitope to be detected.

Finally, the method of the invention requiring one receptor only involves advantages as regards costs and standardisation.

On the basis of the surprising finding according to the invention that particular antigens from said microorganisms have an epitope structure after passage through the intestine that is essentially consistent to detect, a second embodiment must also be considered essential to the invention. This embodiment is based on the fact that different receptors bind to different epitopes of the same antigen. Here, the term

"essentially" means that the epitope(s) and thus a corresponding infection with the microorganism can be detected in more than 70%, preferably at least 75%, more preferably more than 85%, particularly preferred more than 90%, even more preferably more than 95% and most preferably more than 98% of the infected individuals. Ideally, infections are detected in 100% of the infected individuals.

According to the invention, it was surprisingly found that by means of only one single receptor which specifically binds an epitope of an antigen of an acid resistant microorganism, or two receptors which specifically bind two epitopes of the same antigen an infection with these bacteria/pathogens can be diagnosed in a relatively reliable way. The invention includes embodiments in which other epitopes having said properties are recognised by other receptors, for instance, by monoclonal antibodies or fragments or derivatives thereof or aptamers. The latter embodiments are suitable for further increasing the reliability of the diagnosis. Advantageously, these other receptors may be antibodies, fragments or derivatives, which specifically recognise urease, preferably β-urease, the 26 kDa protein or Hsp 60, all preferably from *H. pylori*. The detection of one or more of these proteins/protein fragments may be carried out in the same test or in an independent test with a different part of the same sample.

The results of the invention are surprising mainly because the state of the art had taught away therefrom. In the case of *H. pylori,* for example, it was found that main antigens do not show the desired specificity and sensitivity in ELISA; cf. Newell et al., Serodiag. Immunother. Infect. Dis. **3** (1989), 1-6. Moreover, EP-A- 0 806 667 teaches that it is not possible to reliably detect *H. pylori* infections with receptors, such as monoclonal antibodies due to the genetic variability of *H. pylori* strains.

Compared to the aforementioned state of the art, the method of the invention is of advantage mainly since it permits a relatively reliable diagnosis with only one receptor. In ELISA, for instance, pairs of receptors, such as antibodies, fragments, derivatives thereof or aptamers are used for detection, with the two receptors of the pair binding the same or different epitopes on the same antigen. *H. pylori* catalase, for example, forms multimeric structures of several identical subunits. Therefore, in ELISA or other assays, the same receptors can be used both as catching receptors and detection receptors. Another advantage of the method of the invention is the fact that it is a direct and non-invasive method, which increases the above-mentioned advantages for patient and the reliability for the stage of the disease to be determined.

In a preferred embodiment the acid-resistant microorganism is an acid-resistant bacterium.

A number of acid-resistant bacteria have been known in the state of the art. In a particularly preferred embodiment the acid-resistant bacterium belongs to the genus Helicobacter, Campylobacter or the genus Mycobacterium.

In another particularly preferred embodiment the bacterium is a bacterium belonging to the species *Helicobacter pylori, Helicobacter hepaticum, Campylobacter jejuni* or a bacterium belonging to the species *Mycobactericum tuberculosis*.

In another particularly preferred embodiment the receptor(s) is/are (an) antibody (antibodies), (a) fragment(s) or (a) derivative(s) thereof or (an) aptamer(s).

Within the meaning of the present invention, "fragments" or "derivatives" of monoclonal antibodies have the same binding specificity as the monoclonal antibodies. Such fragments or derivatives can be produced according to standard methods; cf. for example Harlow and Lane, "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, USA, 1988. Examples of fragments include Fab-, $F(ab')_2$ or Fv-fragments. scFv fragments are examples of derivatives. Derivatives can also be chemically produced substances having the same binding properties as the antibodies or improved binding properties. Such substances can be generated, for instance, by peptidomimetics or by different cycles of phage display and subsequent selection as to improved binding properties. According to the invention, aptamers are nucleic acids such as RNA; ssDNA (ss = single-stranded), modified RNA or modified ssDNA, which bind a large number of target sequences having high specificity and affinity. The term "aptamer" has been known and defined in the prior art, for example, in Osborne et al., Curr. Opin. Chem. Biol. **1** (1997), 5-9 or in Stull and Szoka, Pharm. Res. 12 (1995), 465-483.

The term "antigen-antibody complex" within the meaning of the present invention does not only comprise complexes the antigen forms with the native antibody, but also those which it forms with the fragments or derivatives thereof.

The invention includes embodiments in which only monoclonal antibodies or fragments or derivatives thereof or only aptamers are used as well as embodiments in which different types of detection reagents are used in one test. Hence, it is possible for a first monoclonal antibody to be used with a second antibody derivative or a first aptamer to be used with a second antibody fragment, to name only two

examples. In this respect, the terms "first" and "second" refer to the first and the second detection reagent. This, however, does not mean that two antibodies, derivatives or fragments thereof or two aptamers are always used.

The use of monoclonal antibodies, fragments or derivatives thereof or of aptamers ensures easy maintenance of a standard in the reliability of the diagnosis method, which means a great advantage compared to diagnosis methods that have been known so far and that have been introduced for this purpose. Moreover, it is no longer necessary to keep immunizing and subsequently testing new test animals as is required, for instance, in the method according to EP-A 0 806 667.

In another preferred embodiment the antigen is the antigen of a catalase, preferably from *H. pylori*. The catalase has the special advantage that it could be detected in all acid-resistant bacteria known so far. According to the invention it was found, as another advantage, that the catalase is very resistant to digestion in the intestinal tract, which simplifies detection of significant amounts. After all, the catalase or fragments thereof is/are still present in a superior structure, for instance in tetrameric form, after having passed the intestine, which facilitates detection with one receptor type only.

According to the invention, it has surprisingly been found that in a population of mammals, in particular human patients, whose stools had been tested for infections with acid-resistant bacteria, essentially all members of this population showed consistently recurring catalase epitopes in the stool, so that it is very likely to make a relatively reliable diagnosis with only one corresponding receptor, preferably monoclonal antibodies, fragments or derivatives thereof or aptamers. In particular, since the catalase has a tetrameric antigenic structure, this diagnosis can advantageously be made, for instance, in ELISA or in similarly arranged solid systems.

The catalase is particularly preferred to be the catalase of *H. pylori*.

In another preferred embodiment, the antigen is a metalloproteinase, particularly preferably the metalloproteinase from *H. pylori*.

In another preferred embodiment, the antigen is a urease, preferably from *H. pylori*.

In another preferred embodiment, additional use is made of a mixture of receptors for the detection, with the mixture of receptors having the function of a catcher of the

antigen if the receptor is used as detector of the antigen, and the mixture having the function of a detector of the antigen if the receptor is used as catcher of the antigen. This embodiment of the invention permits a particularly reliable diagnosis, especially, if the antigen is not present in a dimeric or multimeric conformation after passage through the intestine. This embodiment makes it possible for only one of the two receptor types used in the majority of the standardized immunological detection methods to be a monoclonal antibody, while, for instance, the second receptor type may be a polyclonal serum.

In a particularly preferred embodiment, the mixture of receptors is a polyclonal antiserum.

In an additionally particularly preferred embodiment, the polyclonal antiserum against a lysate of the microorganism, preferably *H. pylori,* was obtained.

In another particularly preferred embodiment the lysate is a lysate with an enriched antigen.

In another preferred embodiment, the lysate is a lysate with depleted immunodominant antigen.
The two aforementioned embodiments also include the fact that the lysate is a lysate with enriched antigen, preferably with enriched catalase and with depleted immunodominant antigen, preferably mainly antigenic urease. In particular, said combination offers the possibility of obtaining a high immunization yield, which is especially suitable for the method of the invention. A way of carrying out corresponding enrichment and depletion methods is described in more detail in the Examples.

The polyclonal antiserum against a purified or (semi) synthetically produced antigen was obtained according to another particularly preferred embodiment, preferably from *H. pylori.*

According to the invention, the receptors, preferably the monoclonal antibodies, fragments or derivatives thereof or the aptamers can recognise and specifically bind linear or conformation epitopes. In another preferred embodiment, at least one of the receptors binds a conformation epitope.

In a particularly preferred embodiment, all receptors bind conformation epitopes.

In a particularly preferred embodiment, the heavy chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following three CDRs:

CDR1: NYWIH
CDR2: YINPATGSTSYNQDFYD
CDR3: EGYDGFDS

In another particularly preferred embodiment, the DNA sequence encoding the heavy chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: AACTACTGGA TTCAC
CDR2: TACATTAATC CTGCCACTGG TTCCACTTCT TACAATCAGG ACTTTCAGGA C
CDR3: GAGGGGTACG ACGGGTTTGA CTCC

In another particularly preferred embodiment, the light chain of the antibody [HP25.2m/2H10] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: SASSSVNYMY
CDR2: DTSKLAS
CDR3: QQWSSNPYT

Furthermore, in another particularly preferred embodiment, the DNA sequence encoding the light chain of this antibody [HP25.2m/2H10] has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: AGTGCCAGCT CAAGTGTAAA TTACATGTAC
CDR2: GACACATCCA AATTGGCTTC T
CDR3: CAGCAGTGGA GTAGTAATCC GTACACG

In a particularly preferred embodiment, the heavy chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: DTYVH
CDR2: KIDPANGKTKYDPIFQA
CDR3: PIYYASSWFAY

In another particularly preferred embodiment, the DNA sequence encoding the heavy chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: GACACCTATGTGCAC
CDR2: AAGATTGATCCTGCGAATGGTAAAACTAAATATGACCCGATATTC CAGGCC
CDR3: CCCATTTATTACGCTAGTTCCTGGTTTGCTTAC

In another particularly preferred embodiment, the light chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: KASQDVGTSVA
CDR2: WTSTRHT
CDR3. QQYSSSPT

Moreover, in a particularly preferred embodiment, the DNA sequence encoding the light chain of the antibody [HP25.6m/1B5] binding a catalase epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: AAGGCCAGTCAGGATGTGGGTACTTCTGTTGCC
CDR2: TGGACATCCACCCGGCACACT
CDR3: CAGCAATATAGCAGCTCTCCCACG

In another preferred embodiment, the antibody specific to β-urease is the antibody which has been generated by the hybridomas HP8m/4H5-D4-C9 or HP9.1m/3C2-F8-E2 deposited with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ) on June 23, 1998 in accordance with the Statutes of the Budapest Treaty under the accession numbers DSM ACC2360 or DSM ACC2362. The antibody specific to β-urease [HP8m/1H5-G2-B4] which is described in the Figures is produced by a daughter clone of the deposited hybridoma HP8m/4H5-D4-C9. Both antibodies produced by the mother and the daughter clone are encoded by identical DNA sequences and have the same properties.

In another particularly preferred embodiment of the method of the invention, the heavy chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: GFTFSSHFMS
CDR2: SISSGGDSFYPDSLKG
CDR3: DYSWYALDY
or:
CDR1: GYAFSTSWMN
CDR2: RIYPGDGDTNYNGKFKG
CDR3: EDAYYSNPYSLDY

In another particularly preferred embodiment, the DNA sequence encoding the heavy chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: GG CTACGCATTC AGTACCTCCT GGATGAAC
CDR2: CGGATTTATC CTGGAGATGG AGATACTAAC TACAATGGGA AGTTCAAGGG C
CDR3: GAG GATGCCTATT ATAGTAACCC CTATAGTTTG GACTAC
or:
CDR1: GG ATTCACTTTC AGTAGCCATT TCATGTCT
CDR2: TCCATTAGTA GTGGTGGTGA CAGTTTCTAT CCAGACAGTC TGAAGGGC

CDR3: GACTAC TCTTGGTATG CTTTGGACTA C

In another particularly preferred embodiment of the method of the invention, the light chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

CDR1: RASQSIGTRIH
CDR2: YGSESIS
CDR3: QQSNTWPLT
or:
CDR1: HASQNINVWLS
CDR2: KASNLHT
CDR3: QQGRSYPLT

In addition, the DNA sequence encoding the light chain of the said antibody preferably has the following CDRs:

CDR1: A GGGCCAGTCA GAGCATTGGC ACAAGAATAC AC
CDR2: TAT GGTTCTGAGT CTATCTCT
CDR3: CAACAA AGTAATACCT GGCCGCTCAC G
or:
CDR1: C ATGCCAGTCA GAACATTAAT GTTTGGTTAA GC
CDR2: AAG GCTTCCAACT TGCACACA
CDR3: CAACAG GGTCGAAGTT ATCCTCTCAC G

In addition, it is preferred that the heavy and light chains having said CDRs occur together with one antibody, fragment or derivative thereof, which specifically binds the catalase or the β-urease or a fragment thereof, preferably from *H. pylori*. Yet, the invention also comprises embodiments in which these heavy or light chains are combined with other light or heavy chains, wherein these binding properties may essentially be maintained or improved. Corresponding methods have been known in the prior art. Particularly preferred antibodies have in the variable regions of the light and heavy chains the amino acid sequences shown in Figures 1 and 2, 3 and 4, 5 and 6 or 7 and 8 or the regions are encoded by they DNA sequences shown therein.

According to methods known in the state of the art, the CDRs may be integrated in various FRs (framework regions).

In a preferred embodiment, the following steps are carried out with the stool sample before incubation with the antibodies: the stool sample is resuspended in a resuspension buffer at a ratio of 1:3 to 1:25, preferably of 1:10, particularly preferably 1:5 and mixed on a vortex mixer. An example of a sample buffer is: 150 mM PBS, 0.1% SDS. In a preferred embodiment, the resuspension buffer consists of 150 mM PBS, 0.5% animal serum, 0.1% detergent. The animal serum can be selected from cow, mouse, rat or pig and the detergent can be selected from a group of ionic (preferably Tween 20) and non-ionic detergents (preferably SDS) or amphoteric ionic detergents (particularly preferably Chaps).

In another embodiment, the detection according to the invention may also be used for the detection of *H. pylori* in gastric gas, breath condensate, saliva, tooth plaque, mucous smear, biopsies, whole blood or serum. Breath gases can be obtained by giving the patient cold CO_2-containing drinks causing the release of gastric gas in the form of "burping". Said gas can be collected in suitable containers or breath condensate can be recovered in a manner known to the skilled person, e.g. by means of a device according to DE 19718925 or a device according to DE 19505504. The condensates obtained in such a way can then be introduced in a liquid form into the test of the invention with all the steps of the method of the invention as has been described earlier [...][1], except that a sample as described herein is used instead of a stool sample. Tooth plaque and mucous smear can be obtained according to methods known in the state of the art and can, like saliva, whole blood and serum, be used for the detection according to the invention in appropriate concentrations as well as with modifications of the resuspension buffer.

In another preferred embodiment, the formation of the at least one antigen-receptor complex/antigen-receptor-receptor-mixture complex in step (b) is detected by means of an immunologic method.

In another preferred embodiment, the formation of the at least one antigen-receptor complex/antigen-receptor-receptor-mixture complex in step (b) is detected by means of ELISA, RIA, Western blot or an immunochromatographic method. Such methods are basically known in the state of the art; cf. Harlow and Lane, loc. cit.

[1] Translator's note: sentence incomplete. Should probably read. "being carried out"

In a particularly preferred embodiment of the method of the invention, in the immunologic method, in particular in RIA or ELISA, the same receptor is used for both binding to the solid phase and detecting the epitope. While the catcher receptor can be bound to the solid phase, e.g. a micro-titre plate, in unmodified form, the receptor used for detection may optionally be labelled. On the other hand, said receptor may not be labelled and the epitope of the microorganism, preferably the bacterial epitope, may be detected via a third labelled receptor, this receptor preferably being an antibody, fragment or derivative thereof or an aptamer, or a species-specific or Ig class-specific antibody or a corresponding aptamer. Labellings of antibodies, e.g. with radioactive or fluorescent markers are known in the state of the art; cf. Harlow and Lane, loc. cit. The same applies to aptamers. The above-described embodiment is particularly suitable for detecting the catalase which may optionally be present as a tetramer after passage through the intestine. In this embodiment, of course, combinations of antibodies, fragments, derivatives and aptamers can also be used, e.g. combinations of antibodies etc. which bind to different epitopes of the same antigen.

A three-step ELISA is a procedure which comprises the steps of coating the ELISA plate with the catching antibody, adding the sample and the conjugate (e.g. labelled detection antibodies) as well as washing steps in between. The one-step ELISA differs from the three-step ELISA in that the sample and the conjugate are added and applied onto an ELISA plate pre-coated with the catching antibody in one step.

In another preferred embodiment of the method of the invention, the monoclonal antibody is a murine antibody.

In addition, in another preferred embodiment, the receptors are fixed to a support.
When carrying out routine checks, it is of particular advantage to fix the receptors, preferably the antibody, fragments or derivatives thereof or the aptamers to a support. Moreover, the combination antibody-support/aptamer-support may be packaged as a tool set or in the form of a kit.

In another particularly preferred embodiment, the material of the support is a porous support material.
In another particularly preferred embodiment, the support material is a test strip.

In addition, in a preferred embodiment, the support material consists of cellulose or a cellulose derivative.

The mammal whose stool, gastric gas, breath condensate, etc. can be analysed by means of the method of the invention may be an animal, e.g. a domestic animal such as a cat or a dog, a useful animal such as a pig or another kind of animal such as a mouse, a tiger, a gerbil or a ferret.

In another preferred embodiment, the mammal is a human.

In another preferred embodiment, the method of the invention is an automated method. An automated method may, for instance, be carried out by means of a robot, with the robot carrying out some or all steps of the procedure. Corresponding robots are known in the state of the art.

Furthermore, the invention relates to a monoclonal antibody, a fragment or derivative thereof having a V region which has a combination of the aforementioned CDRs or which is produced by one of the aforementioned hybridomas.

In this case, a monoclonal antibody, a fragment or a derivative thereof is preferred which has at least one of the V regions depicted in Figures 1 and 2, 3 and 4, 5 and 6 or 7 and 8. Preferably, this antibody has two of the V regions shown in Figures 1 and 2, 3 and 4, 5 and 6 or 7 and. Moreover, these V regions are preferred to be encoded by the DNA sequences shown in Figures 1 and 2, 3 and 4, 5 and 6 or 7 and 8.

In a particularly preferred embodiment of the invention, the monoclonal antibody, the fragment or derivative thereof is a murine antibody or a fragment or derivative thereof or a chimeric, preferably a humanized antibody or a fragment or derivative thereof. The derivative may also be a fusion protein. Furthermore, the antibody is preferred to be labelled, for instance with a colloid, with a radioactive, fluorescent, phosphorescent or chemiluminescent labelling.
The production of chimeric humanized and human antibodies and of the other derivatives has been well known in the state of the art (e.g. Vaughan et al., 1998; Orlandi et al., 1989, Harlow and Lane, loc. cit.).

The invention also relates to an aptamer which specifically binds the same epitope as the monoclonal antibody, the fragment or derivative thereof. Such aptamers can be produced according to methods known in the state of the art.

In addition, the invention relates to an epitope which is specifically bound by one of the above-described antibodies, fragments or derivatives thereof or aptamers.

Furthermore, the invention relates to further antibodies, derivatives or fragments thereof, which specifically bind the epitope of the invention. These antibodies may, for instance, be monoclonal antibodies which can be produced according to standard methods using the epitope as a hapten/component of an antigen.

Moreover, the present invention relates to a diagnostic composition containing at least one receptor, preferably at least one monoclonal antibody, fragment or derivative thereof or aptamers as defined above, optionally fixed to a support material.

Furthermore, the present invention relates to a test device for detecting at least one of the above-defined epitopes, comprising (a) at least one receptor which is preferred to be a monoclonal antibody, fragments or derivatives thereof or an aptamer as defined above, fixed to a support material; (b) a device for preparing and analysing stool samples and optionally, a mixture of receptors as defined above.

A further subject matter of the invention is a test device comprising (a) at least one receptor, preferably a monoclonal antibody, fragments or derivatives thereof or an aptamer as defined above, with the receptor being conjugated with colloidal gold, latex partciles or other colouring particles the size of which typically ranges from 5 nm to 100 nm, preferably from 20 nm to 60 nm (a particle size of 40 nm to 60 nm for gold and of 200 nm to 500 nm for latex is particularly preferred); (b) a device for preparing and analysing stool samples; and optionally (c) a mixture of receptors as defined above.

Furthermore, the present invention relates to a kit containing (a) at least one receptor which preferably is a monoclonal antibody, fragments or derivatives thereof or an aptamer as defined above and which is optionally fixed to a support material; optionally also (b) a device for preparing and analysing stool samples; and optionally (c) a mixture of receptors as defined above.

Alternatively to the devices for preparing and analysing stool samples, the compositions and test devices and kits may also have devices for preparing (if necessary) and analysing gastric gases, breath condensate, saliva, etc.

The invention also relates to a composition containing at least one of the aforementioned receptors, optionally in combination with a pharmaceutically acceptable support and/or a diluent. The composition is preferred to be a pharmaceutical preparation.

The person skilled in the art knows examples of appropriate pharmaceutically acceptable supports. These include phosphate-buffered saline solutions, water, emulsions such as oil/water emulsions, different kinds of detergents, sterile solutions, etc. Pharmaceutical preparations comprising such supports may be formulated by means of known, conventional methods. These pharmaceutical preparations can be administered to an individual in an appropriate dose ranging, for example, from 1 μg to 100 mg per day and patient. There are various forms of administration, e.g. intravenous, intraperitoneal, subcutaneous, intramuscular, local or intradermal. The physician in charge will choose the dosage in accordance with the clinical factors. The person skilled in the art knows that the dosage depends on various factors such as size, body surface, age, sex or general state of the patient, but it also depends on the specific pharmaceutical preparation that is administered , the duration and the kind of application and on other pharmaceutical preparations which are possibly administered at the same time.

Finally, the invention relates to a package containing the diagnostic composition of the invention, the test device of the invention or the kit of the invention.
The components of the diagnostic composition of the invention, the test device of the invention and/or the kit of the invention may be packed in containers such as vials or tubules, optionally in buffers and/or solutions. Possibly, one or more components may be packed in one container.

The Figures illustrate:

Fig. 1: A cloned DNA sequence coding for the V region of the heavy chain of monoclonal antibody [HP25.2m/2H10] specific to catalase. The encoded amino acid sequence is shown in a single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 2: A cloned DNA sequence coding for the V region of the light chain of a monoclonal antibody [HP25.2m/2H10] specific to catalase. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 3: A cloned DNA sequence coding for the V region of the heavy chain of a monoclonal antibody [HP25.6m/1B5] specific to catalase. The encoded amino acid

sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 4: A cloned DNA sequence coding for the V region of the light chain of a monoclonal antibody [HP25.6m/1B5] specific to catalase. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 5: DNA sequence coding for a light chain of a first monoclonal antibody (DSM ACC2360) specific to urease. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 6 DNA sequence coding for a heavy chain of a first monoclonal antibody (DSM ACC2360) specific to urease. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 7: DNA sequence coding for a light chain of a second monoclonal antibody (DSM ACC2362) specific to urease. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 8: DNA sequence coding for a heavy chain of a second monoclonal antibody (DSM ACC2362) specific to urease. The encoded amino acid sequence is shown in single-letter code. The CDR regions 1-3 determined according to Kabat et al. are underlined.

Fig. 9: Course of eradication treatment of an *H. pylori* positive patient after taking Omeprazol, Metronidazol and Clarithromycin.

The Examples illustrate the invention.

Example 1: Isolation of *H. pylori* antigens

Cultivation of *H. pylori*

H. pylori (strain NCTC 11637) was plated in petri dishes on Wilkins chalkers agar adding 10% horse blood and Amphotericin B, Vancomycin and Cefsoludin (Sigma Chemicals) and incubated in an microaerophile atmosphere (Anaerocult GasPAk, Merck) at 37°C for 3 or 4 days. The content of 2 dishes was suspended in a 1 l-bottle (Schott) in 350 ml of BHIB medium adding the antibiotics as above, the medium was fumigated for 10 min with a gas mixture of 10% CO_2, 5 % O_2, 85% N_2 and the bottle was sealed. The culture was shaken on a rotary shaker for 2 days at 37°C. Then, the content of the bottle was put aseptically in a 10 l-bottle and filled up with 4,7 l BHIB-medium. It was incubated on a rotary shaker for another 2 days at 37°C. Subsequently, the whole volume was centrifuged at 5,000 g for 15 min, the supernatant was decanted and the bacteria pellet was weighed. In order to store the pellet, it was resuspended in a physiological saline solution adding 15% glycerine at a ratio of 2:1 (w/v) and frozen at –80°C. In order to check the identity of the cultivated bacteria, a microscopic inspection of the bacteria as well as tests for urease, oxidase and catalase activity were carried out.

Example 2: Preparation of *H. pylori antigens*

Preparation of *H. pylori* lysate

PBS, pH 7.5 was added to *H. pylori* bacteria pellet (Example 1) at a ratio of 1:10 and resuspended on ice. The bacteria cells were sonicated on ice with a small ultrasonic detector (Sonifer, Branson) with an intensity of 25 – 30% for 10 x 60 s with a break of 60 s each. The disrupted bacteria cells were centrifuged 2 x 20 min at 4°C and 10,000 rpm (Sorvall, SS34). The supernatant was used as antigen preparation for the production of polyclonal antisera.

Preparation of *H. pylori* catalase

Disruption buffer (20 mM Tris HCl, pH 7.0, 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 0.05% sodium azide and 10% (v/v) isobutanol) was added to frozen bacteria pellet at a ratio of 1:2 (w/v) and shaken at room temperature (RT) in an overhead shaker until complete thawing and subsequently shaken for another approximately 15 min. After centrifugation at 20,000 rpm (Sorvall, SS-34), 4°C for 20 min, the supernatant was decanted and filtered through a 0.45 μm-filter.

The clear supernatant was diluted with buffer A (20 mM Tris HCl, pH 7.0, 1 mM EDTA, 1 mM PMSF, 0.05% sodium azide) at a ratio of 1:3 and transferred onto a SourceQ column (16/10) (Pharmacia) equilibrated with buffer A. The flow through of the SourceQ column contained the enzyme catalase and was free of *H. pylori* main antigens such as urease, Hsp60 and alkylhydroperoxide reductase.
In order to isolate the katalase, the flow through of the SourceQ column was subjected to a molecular sieve chromatography (Superdex 200) (16/60). The catalase was isolated together with another protein with a size of approx. 150 kDa (neutrophil activating protein, NAP) in about equal shares.
Catalase with a higher purity was obtained when the flow through of the SourceQ-column was put in a 2 M sodium acetate solution, pH 4.9, on 40 mM sodium acetate and was transferred on a SourceS column (8/28). After washing with buffer A to remove the proteins that are not bound, the catalase was eluted with buffer B (40 mM sodium acetate, 1 M NaCl, pH 4.9) using a linear NaCl gradient (buffer A plus 0% to 100% of buffer B). Catalase elutes at approx. 370 mM NaCl.

Example 3: Characterisation of the catalase:

Under reducing conditions in SDS PAGE, the purified protein had a molecular weight of approx. 58 kDa and a purity of ≥ 90%.
In order to identify the isolated protein, a micro sequencing was carried out. The protein was cleaved in SDS PAGE gel with LysC protease. The extracted protein mixture was separated via RP-HPLC. The sequence analysis of the LysC peptide resulted in the following amino acid sequence:

ERLHDTIGESLAHVTHK

This sequence is identical to the corresponding LysC peptide from *H. pylori* catalase (Manos J. et al. (1998) Helicobacter 3 (1), 28-38; Genbank accession no. AAC16068.1).

Example 4: Production of polyclonal and monoclonal antibodies (pab; mab)

Production of polyclonal antisera:

Polyclonal antisera against *H. pylori* lysate, *H. pylori* lysate with depleted main antigens such as urease, Hsp60 and alkylhydroperoxide reductase (cf. Example 2: isolation and purification), *H. pylori* lysate with enriched catalase (for example by adding catalase to the lysate), as well as polyclonal antisera against purified catalase can be obtained by immunizing a selected mammal (e.g. mouse, rabbit, goat, etc.) with the corresponding immunogenic preparations containing the catalase epitope.

The antibodies can be purified by means of protein A affinity chromatography of sera and can be used as catching antibodies in sandwich ELISA (cf. Example 9) for assessing whether the monoclonal antibodies are suitable for antigen detection in the stool of patients.

Polyclonal rabbit antisera were generated by pab Productions (Herbertshausen) from *H. pylori* lysate. By means of protein A affinity chromatography polyclonal antibodies were purified from these antisera and used as catching antibodies in Sandwich ELISA (cf. Example 9) for assessing whether the monoclonal antibodies are suitable for antigen detection in the stool of patients.

Production of monoclonal antibodies:

The monoclonal antibodies were generated according to methods known to the person skilled in the art (Harlow & Lane, 1988; Peters & Baumgarten, 1990).

Immunization

Antigen preparations produced from *H. pylori* lysate (cf. Example 2) were used for immunizing mice (BALB/c x C57/Black, F1 generation, 8-12 weeks old). For basic immunization 50 µg antigen were emulsified with Freund complete adjuvant (Difco) at a ratio of 1:1 and injected intraperitoneally (200 µl/mouse). In booster shots every four months, the mice were given 25 µg antigen each with Freund incomplete adjuvant. An antiserum as positive control in ELISA (cf. fusion screening) was obtained from blood taken retro-orbitally from the mice.

Fusion

Two days after the last immunization, the spleens of the mice were removed and the spleen cells were fused with the myeloma cells P3x63Ag8.653 (ATCC CRL-1580; Kearney et al., 1979) with polyethylene glycol 4000 at a ratio of 5:1. The fused cells were suspened in HAT medium (cloning medium (= RPMI 1640 medium, 20% FCS, 200 U/ml rhIL-6) with hypoxanthine aminopterin thymidine supplement (100x concentrate; Sigma)) and plated in 96-well micro-titre plates with a cell density of 2-6x10^4 cells/well. The hybridomas were cultivated at 37°C, 5% CO_2 and 95% relative humidity.

Fusion screening by means of direct ELISA

Screening of the antibody-containing culture supernatants from colonized dishes (approx. 10 days after the fusion) was carried out in direct ELISA on 96-well micro-titre plates (MaxiSorb, Nunc):
The ELISA plates were coated with 2 µg/ml immunization antigen in carbonate buffer, pH 9.6 (100 µl/well, over night, 5°C). The coating solution was sucked off and binding sites that were still free were blocked with 2% skimmed-milk powder in PBS (w/v) (200 µl/well, 1 hour, room temperature). After washing the plate twice with PBS, pH 7.3 with 0.025% Tween 20 (v/v), the culture supernatants of the primary clones were pipetted undiluted in the wells (100 µl/well) and the plates were incubated for 1-2 hours at room temperature. The antiserum was used as a positive control, the medium as a negative control. After washing again, the detection of the bound

antibodies was carried out with a peroxidase-labelled secondary antibody (rabbit-anti-mouse Ig-POD (DAKO) in PBS with 0.1% bovine serum albumin, 20 min, room temperature). The peroxidase turns the colourless substrate tetramethyl benzidine (TMB, Sigma) into a coloured complex. After washing and knocking the plate four times, the substrate solution (K-Blue, Neogen or citric acid buffer, pH 4.5, with TMB + H_2O_2) was added. After 10 min the reaction was stopped by adding 1 N sulfuric acid. Culture supernatants of clones producing antigen-specific antibodies were significantly coloured compared to the colourless negative culture supernatants.

Establishing and cultivating the hybridomas

Positive clones were recloned twice according to the principle of limiting dilution analysis in order to obtain monoclones (Coller & Coller, 1983). The first recloning was carried out in cloning medium with hypoxanthine thymidine supplement (100x concentrate; Sigma), the second one in cloning medium. The reclones were examined for antigen specificity by means of direct ELISA. In the end, the final clone was adapted to production medium (RPMI 1640 Medium with 5% IgG-reduced FCS) in flat bottles. The cells were cryo-preserved and the culture supernatant was produced for the antibody purification.

Example 5: Characterisation of the antibodies from the culture supernatant

10 clones were selected from a repertoire of 30 specific (producing antibodies against the immunization antigen) clones by means of their good reactivity to stool samples of patients infected with *H. pylori* in Sandwich ELISA (cf. Table 2).

Isotyping

In the culture supernatant isotyping of the monoclonal antibody was carried out with the establishing clones using the isotyping Kit IsoStrip (Roche Diagnostics). The result was: 8 type IgG1-clones and one type IgG2a-clone (cf. Table 2).

Western blot

In Western blot, the culture supernatants were examined for their ability to specifically recognise the immunizing antigen. 15 µg purified antigen per gel were boiled in reducing sample buffer (Laemmli, 1970) and applied to a 12%-SDS polyacrylamide mini gel (8.6 cm x 7.7 cm x 0.1 cm, Biometra). After electrophoretic separation at 25-30 mA, the proteins (antigen) were immobilized on a nitrocellulose by means of semi-dry blot technique.
The membrane was blocked with 2% skimmed-milk powder in PBS (30 min, room temperature) and washed three times for 5 min with TBS/Tween 20 (0.2%). For the following incubation step, the membrane was clamped in an Accutran cross blot screening unit (Schleicher and Schüll) using a grid plate with 34 cross channels. In each of the traces that were formed, 250 µl of TBS/Tween 20 and 250 µl of the hybridoma culture supernatants to be tested are added. Incubation was carried out while shaking for 2 h at room temperature.
After washing three times[2] TBS/Tween 20, the membrane was incubated for 1 h with the POD-conjugated secondary antibody (rabbit-anti-mouse Ig-POD, DAKO). The membrane was washed three times and the immune complex was visualised by adding the 3,3-diaminobenzidine substrate solution (DAB, Sigma). The protein bands binding the antibodies were subsequently visualised by an insoluble peroxidase substrate.
6 hybridoma culture supernatants exhibited a band that corresponds to the catalase (58 kDa), 3 were negative in Western blot, however, showed a positive reaction with native antigen in ELISA. They are likely to recognise a conformation epitope. Table 2 shows a summary of the results.

Example 6: Purification of monoclonal antibodies from hybridoma culture supernatants

The purification of mab from serum-free hybridoma culture supernatants was carried out by means of a modified protein-G affinity chromatography (Pharmacia Biotech, 1994).
The filtered (0.45 µm) culture supernatants were conducted directly over a protein G matrix. The detection of the protein in the flow through or in the eluate was carried out via measuring the optical density at 280 nm. After washing with 150 mM PBS, pH 7.2, until the detector background value, elution was conducted with 0.1 M

[2] Translator's note: word missing. Should probably read: "with"

glycine/HCl, pH 3.3. The protein-G matrix was regenerated with 0.1 M glycine/HCl, pH 2.7.

Example 7: Production of conjugates

Coupling of mab to peroxidase (POD) for use in ELISA

The mab were coupled externally to (POD). Poly-POD conjugates were obtained from MicroCoat (Bernried, Germany), HPR (horseradish peroxidase)-dextran conjugates were obtained from DAKO (Copenhagen, Denmark).

Coupling of mab to biotin for use in ELISA

After purification, the monoclonal antibodies are biotinylated so that they can be used as detection antibodies in ELISA. Coupling of the monoclonal antibodies to biotin and POD was carried out according to known methods (Harlow & Lane, 1988).

The monoclonal antibodies were conjugated at a concentration of approx. 1-2 mg/ml. Before coupling, the antibodies were rebuffered by dialysis in 0.1 M sodium acetate buffer, pH 8.3 and 0.1 M sodium hydrogen carbonate buffer, pH 8.3. For each 1 mg of antibodies 50 μg N-hydroxysuccinimidobiotin (NHS-d-biotin; Sigma) was pipetted and mixed in DMSO. The mixture was incubated for one hour at room temperature. Then, the biotinylated antibodies were freed from uncoupled NHS-d-biotin by extensive dialysis against 0.15 M PBS, 0.05% NaN_3, pH 7.5.

Coupling of mab to colloidal gold for use in immunological rapid tests

The monoclonal antibody (mab) was conjugated to colloidal gold for use in immunological rapid tests. This is carried out according to standard methods (Frens, 1973; Geoghegan and Ackerman, 1977; Slot et al., 1985). For the production of colloidal gold, 200 ml of a 0.01% gold chlorite- ($HAuCl_4$)-solution is heated until boiling and reduced during further boiling by adding 2 ml of 1% sodium citrate ($Na_3C_6H_5O_7$).

For coupling mab to colloidal gold, an amount of IgG necessary for stabilisation is mixed with the gold solution and incubated for 15 min at room temperature. The optimal IgG concentration and the suitable pH value for coupling were determined individually for each mab. Polymers or protein, e.g. bovine serum albumine (BSA) are added in a concentration of 1% to the coupling preparation in order to stabilise the gold IgG conjugate. Gold colloid that was not coupled to IgG and free IgG were subsequently removed from the coupling preparation by centrifugation from the gold IgG conjugate. For storage, preferably at 4°C, 0.05% of NaN_3 was added to the solution buffer of the gold IgG conjugate.

Example 8: Characterisation of the purified monoclonal antibody

Characterisation of antibody-antigen interactions by means of surface plasmon resonance spectroscopy (SPR spectroscopy)

By means of SPR spectroscopy, it is possible to determine the affinity constants of the monoclonal antibodies. Thus, suitable antibodies for the development of ELISA and rapid tests can be found.

Conduction of the surface plasmon resonance spectroscopy on the Pharmacia BIAcore

All steps were carried out on a Pharmacia Biacore Processing Unit CA 186 according to the manufacturer's instructions (BIAcore Methods Manual).
Catalase was immobilized through amine coupling on the dextrane matrix of the BIAcore CM5 sensor chip. For the activation of the dextrane matrix 45 µl of a 1:1-mixture of 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) solution was conducted over the sensor chip at a flow rate of 5 µl/min. Then, the catalase (35 µl; 50 µg/ml in 10 mM sodium acetate pH 5.0) was bound to the dextrane matrix. The remaining NHS ester was deactivated with 1 M ethanolamine (35 µl). Catalase that was not covalently bound to the dextrane matrix was removed by regenerating the sensor chip with HCl (10 mM; 15 µl).

By adding the catalase-specific monoclonal antibodies, these were made react with immobilized catalase and the mass attachment to the detector was measured. Antibody solutions in different concentrations ranging from 20 to 670 nM were used. They were injected via the catalase immobilized on the sensor chip at a flow rate of 25 µl/min each.

Results:

The values for the rate constants of the adsorption (k_{on}) and desorption (k_{off}) of the antibody could be calculated by means of the time course of the resonance signal (BIAevaluation software 3.0). 6 monoclonal antibodies against catalase were tested as to their affinities:

Table 1: results of the affinity determination of catalase mab

mab	k_{on} [M^{-1} s^{-1}]	k_{off} [s^{-1}]	K_D [M]
HP25.2m/2H10	1.44E+05	3.90E-05	2.71E-10
HP25.6m/1G4	1.41E+05	2.52E-05	1.79E-10
HP25.6m/1B5	5.67E+04	3.86E-05	6.81E-10
HP25.6m/4E3	4.92E+04	5.96E-05	1.21E-09
HP25.6m/1A5	3.91E+04	4.77E-05	1.22E-09
HP25.6m/1H4	7.12E+04	4.12E-05	5.79E-10

$K_D = k_{off} : k_{on}$

Selection of antibody pairings for use in ELISA at human stool

Antibodies showing the lowest detection limit when the culture supernatant was measured were determined by means of surface plasmon resonance epitope overlaps and the affinity constants were measured. The combinations which were promising (no epitope overlaps, high rate constant for adsorption, low rate constant for desorption) were tested for their antigen detection-limit in sandwich stool ELISA.

Example 9: Screening of mab culture supernatants on patients' samples (mixed polyclonal/monoclonal system)

The monoclonal antibodies exhibiting a specific antigen recognition by means of direct ELISA (Example 4) were analysed as culture supernatants in sandwich ELISA as to their patient recognition and antigen detection-limit.
As internal development samples, stool samples were used the infection status of which (groups 0 and 4) was determined by means of histological analyses and/or ^{13}C urea breath test. Patients of group 0 showed a *H. pylori*-negative result, patients of group 4 showed a *H. pylori*-positive result in the reference test.
The ELISE plates (micro-titre plate MaxiSorb; Nunc) were coated overnight at 5°C with 100 µl of polyclonal rabbit anti-catalase antibody or with a polyclonal rabbit anti-*H. pylori* antibody (pab; approx. 20 µg IgG/ml 0.1 M carbonate buffer, pH 9.5). In order to block the binding sites that were still free, 200 µl of 150 mM PBS pH 7.2 with 0.2% fish gelatine (w/v) were pipetted per well and incubated at room temperature for 30 min. Then, the ELISA plate was washed twice with 250 µl PBS adding 0.025% Tween 20 (washing buffer 1). Human stool was suspended with 150 mM PBS at a ratio of 1:10 (w/v) adding 2% of skimmed-milk powder and 1 mM EDTA.
For the determination of the antigen detection-limit, a *H. pylori*-negative stool suspension was mixed with 50 ng/ml catalase (cf. Example 3) and diluted with a *H. pylori*-negative stool suspension in 1:2 steps. 100 µl of the stool suspension per well were incubated for one hour (double determination in the case of patients' samples). The ELISA plate was knocked out, rinsed with washing buffer 2 (PBS with 0.2% of Tween 20) and washed 4 times with washing buffer 2. Then, 100 µl culture supernatant of hybridomas (1:5 diluted in PBS) was added and incubated at room temperature for 60 min. The bound antibodies were detected by adding a peroxidase-conjugated secondary antibody (rabbit-anti-mouse IgG-POD, DAKO). In the next step, the peroxidase turns the added colourless POD substrate tetramethylbenzidine (TMB, Sigma) into a blue product. After 5 to 10 minutes, preferably after 10 min, the enzyme reaction was stopped by adding 1 N sulfuric acid (100 µl/well). The intensity of the colour reaction was measured in the ELISA reader (MWG Spektral). Measurement was carried out at 450 nm against the reference wave length of 620 nm, preferably of 630 nm. Before the detection antibody or the substrate solution were added, the ELISA plate was washed three to four times with washing buffer 1.
An extinction value which was bigger than or equal to the double of the zero-value (*H. pylori*-negative stool sample without addition of antigens) could still be detected was determined to serve as detection limit.

Using a polyclonal catching antibody which is directed against *H. pylori* lysate, the monoclonal antibody HP25.2m/2H10 had a sensitivity of 68% (out of 25 positive samples 17 were detected correctly) and a specificity of 82% (out of 17 HP-negative samples, 3 were false-positive) in sandwich ELISA. The patient recognition of further monoclonal antibodies (culture supenatants) can be seen from Table 3.

Table 2: HP25.2m/2H10: sensitivity and specificity in sandwich ELISA (catching antibody pab against *H. pylori*)

stool sample	patient's infection status	catching ab: pab against HP detection ab: HP25.2m/2H10 (culture supernatant) $OD_{450-630}$	evaluation: cut off: 0.1: $OD_{450-630} = 0.1$
CX0010	POSITIVE	0.25	positive
CX1014	POSITIVE	0.75	positive
CX1029	POSITIVE	0.18	positive
CX1038	POSITIVE	0.09	negative
CX1052	POSITIVE	0.11	positive
CX2008	POSITIVE	0.63	positive
CX2009	POSITIVE	0.32	positive
CX2016	POSITIVE	0.07	negative
CX2019	POSITIVE	0.59	positive
CX2029	POSITIVE	0.52	positive
CX0213	POSITIVE	0.04	negative
CX294-1	POSITIVE	0.14	positive
CX3098	POSITIVE	0.13	positive
CX3146	POSITIVE	0.05	negative
CX3148	POSITIVE	0.08	negative
CX3234	POSITIVE	0.18	positive
CX4003	POSITIVE	0.17	positive
CX4006	POSITIVE	0.25	positive
CXT001	POSITIVE	0.23	positive
CXT002	POSITIVE	0.53	positive

CXT003	POSITIVE	0.12	positive
CXT004	POSITIVE	0.03	negative
CXT005	POSITIVE	0.03	negative
CXT006	POSITIVE	0.31	positive
CXT007	POSITIVE	0.08	negative
CX1008	NEGATIVE	0.29	positive
CX1031	NEGATIVE	0.08	negative
CX1049	NEGATIVE	0.7	positive
CX1051	NEGATIVE	0.09	negative
CX0142	NEGATIVE	0.03	negative
CX0185	NEGATIVE	0.03	negative
CX0189	NEGATIVE	0.08	negative
CX0193	NEGATIVE	0.03	negative
CX2010	NEGATIVE	0.08	negative
CX2018	NEGATIVE	0.09	negative
CX0220	NEGATIVE	0.03	negative
CX0231	NEGATIVE	0.03	negative
CX0258	NEGATIVE	0.02	negative
CX3008	NEGATIVE	0.09	positive
CX3011	NEGATIVE	0.08	negative
CX3033	NEGATIVE	0.07	negative
CX3035	NEGATIVE	0.09	negative

Abbreviations: pab: polyclonal antibody; HP: *H. pylori*

Table 3: Characterisation of the monoclonal antibodies against catalase

fusion/clone	**isotype**	**WB (ag)**	**NWG (ng/ml)**	**stool samples that were correctly detected**	
				pos. samples	neg. samples
HP25.2m/2H10	IgG2a, κ	+	1.5	17 out of 25	14 out of 17
HP25.6m/1G4	IgG1, κ	+	1.5	4 out of 5	2 out of 2

HP25.6m/1B5	IgG1, κ	+	3-6	3 out of 5	2 out of 2
HP25.6m/1H4	IgG1, κ	+	3-6	2 out of 5	2 out of 2
HP25.6m/4E3	IgG1, κ	+	6	2 out of 5	2 out of 2
HP25.6m/1A5	IgG1, κ	+	6	2 out of 5	2 out of 2
HP25.6m/5E4	IgG1, κ	-	1.5	1 out of 5	2 out of 2
HP25.6m/4A12	IgG1, κ	-	1.5	1 out of 5	2 out of 2
HP25.6m/5F4	IgG1, κ	-	1.5	1 out of 5	2 out of 2

Abbreviations: ag: antigen; WB: Western blot; NWG: detection limit

Results:

Table 3 summarises the results of the isotype determination, the Western blot analyses, the determination of the detection limits and the detection of monoclonal antibodies (mab) against catalase in patients. The data shows that a good detection of native catalase by means of mab does not correlate with a good detection in patients.
In the mixed polyclonal/monoclonal sandwich ELISA system the mab HP25.2m/2H10 showed a sensitivity of 68% and a specificity of 82%. The sensitivity and specificity is expected to be improved using purified mab (instead of the culture supernatant) in a merely monoclonal ELISA system. In this case, either a monoclonal antibody directed against the same epitope of the antigen (cf. Example 10) or two different monoclonal antibodies directed against different epitopes of the same antigen (cf. Example 12) can be used as catching and detection antibodies.

Example 10: Detection of *H. pylori* in human stool by means of ELISA (purely monoclonal system)

For the test, stool samples of patients of ten different hospitals or gastroenterological surgeries were at disposal. The *H. pylori* status was determined by means of ^{13}C urea breath test and/or histological analyses of gastric biopsies. The stool samples to be tested were codified so that the laboratory staff did not know about the infection status.

H. pylori stool sandwich ELISA (three-step ELISA)

The ELISA plates (MaxiSorb; Nunc) were coated with 100 µl of an mab solution (2.0 µg HP25.2m/2H10/ml, 0.1 M carbonate buffer, pH 9.5) for 1 hour at 37°C. In order to block the binding sites that were still free, 200 µl 150 mM PBS with 0.2% fish gelatine (w/v) were pipetted per dish and incubated at room temperature for 30 min. Subsequently, they were washed twice with 250 µl washing buffer 1 (PBS with 0.025% Tween). Human stool was suspended with 150 mM PBS at a ratio of 1:10 (w/v) adding 2% of skimmed-milk powder and 1 mM EDTA. In order to determine the antigen detection-limit, purified *H. pylori* catalase was added in known concentrations to the stool suspension of a *H. pylori*-negative patient. The stool sample suspensions were centrifuged off at 7,000 g for 5 min. 100 µl of the supernatant per well were incubated for one hour. The plate was knocked, rinsed and washed four times with washing buffer 2 (250 µl PBS adding 0.2% Tween). Then, 100 µl of a solution of biotin-coupled mab (1 µg/ml HP25.2m/2H10-Bio in PBS; 0.1% BSA) was added and incubated at room temperature for 60 min. The bound antibodies were detected by adding a conjugate of streptavidin with POD (Dianova). In the next step, the POD turns the colourless substrate TMB (Sigma) into a blue product. After five to ten minutes, preferably after 10 min, the enzyme reaction was stopped by adding 1 N sulfuric acid (100 µl/well). The intensity of the colour reaction was measured in the ELISA reader (MWG Spektral). Measurement was carried out at 455 nm against the reference wave length of 620 nm or 630 nm.

Table 4: Detection of *H. pylori* catalase in the stool by means of ELISA using the monoclonal antibody HP25.2m/2H10 as catching and detection antibody

patients	clinical status	HP stool ELISA OD(455-630)
1001	negative	0.069
1002	negative	0.104
1007	negative	0.053
1008	negative	0.042
1010	negative	0.043
1012	negative	0.055
1017	negative	0.052
1021	negative	0.045
1022	negative	0.068
1024	negative	0.036
1025	negative	0.046
1027	negative	0.057
1030	negative	0.061
1031	negative	0.037
1032	negative	0.056
1034	negative	0.048
1035	negative	0.033
1040	negative	0.037
1046	negative	0.046
2002	negative	0.056
2006	negative	0.032
2007	negative	0.027

2010	negative	0.039
2012	negative	0.041
2013	negative	0.049
2014	negative	0.046
2015	negative	0.048
2017	negative	0.050
2018	negative	0.061
2023	negative	0.056
2024	negative	0.051
2028	negative	0.102
2033	negative	0.050
2034	negative	0.077
2043	negative	0.045
3123	negative	0.055
3213	negative	0.119
3214	negative	0.062
3224	negative	0.048
3225	negative	0.065
3236	negative	0.043
4004	negative	0.089
5004	negative	0.079
5007	negative	0.055
5008	negative	0.156
5009	negative	0.076
5010	negative	0.073
5012	negative	0.051
5013	negative	0.057
5017	negative	0.064
5018	negative	0.033
5019	negative	0.017
5020	negative	0.017
5021	negative	0.019
5022	negative	0.020
5024	negative	0.015
5025	negative	0.017
5027	negative	0.022
5028	negative	0.021
5030	negative	0.019
5031	negative	0.014
5033	negative	0.018
5034	negative	0.013
5035	negative	0.018
5036	negative	0.031
5040	negative	0.024
5042	negative	0.026
5046	negative	0.021
5052	negative	0.020
5056	negative	0.523
5057	negative	0.023
5060	negative	0.055
5063	negative	0.022
5064	negative	0.017
5065	negative	0.035
5066	negative	0.024
5067	negative	0.088
5068	negative	0.021
6002	negative	0.078
6005	negative	0.019
6008	negative	0.013
6019	negative	0.034
7005	negative	0.025
7006	negative	4.556
7009	negative	0.030
7013	negative	0.024
8004	negative	0.023
8047	negative	0.021
213	positive	0.879
294	positive	4.097
444-1	positive	0.201
1003	positive	0.475
1013	positive	4.087
1014	positive	0.105
1015	positive	2.469
1028	positive	0.096
1029	positive	4.466
1037	positive	2.485
2001	positive	0.083
2003	positive	0.817
2005	positive	1.508
2008	positive	4.247
2009	positive	1.597
2016	positive	2.651
2022	positive	0.135
2029	positive	3.953
2032	positive	3.400
2035	positive	3.384
2039	positive	0.053
2040	positive	4.602
2041	positive	0.200
2042	positive	4.592
3146	positive	1.742
6014	positive	2.572
3149	positive	0.989
3153	positive	4.590
3570	positive	4.567
3577	positive	4.566
3215	positive	4.540
3219	positive	4.486

3220	positive	4.518
3231	positive	4.706
3234	positive	4.567
3235	positive	4.616
3241	positive	3.671
3243	positive	4.582
4003	positive	4.700
4005	positive	0.401
4006	positive	4.694
4018	positive	4.142
4019	positive	2.366
4020	positive	1.468
5001	positive	4.490
5002	positive	3.917
5003	positive	4.321
5006	positive	4.826
77	positive	0.067
5011	positive	0.071
53	positive	4.773
70	positive	1.084
5016	positive	0.101
68	positive	4.611
5069	positive	1.079
CXT 5	positive	0.602
5072	positive	4.151
5075	positive	4.307
5076	positive	4.516
CXT 4	positive	0.268
5078	positive	1.022
6001	positive	4.441
6004	positive	4.296
CXT 3	positive	2.126
6018	positive	4.656
6020	positive	0.427
7001	positive	2.717
CXT 2	positive	4.479
7002	positive	4.143
7003	positive	0.149
7004	positive	4.543
CXT 1	positive	0.953
8026	positive	0.025
8033	positive	0.784

67	positive	0.589
5029	positive	0.675
64	positive	1.785
58	positive	0.304
5039	positive	3.391
CXT 13	positive	3.785
6013	positive	1.972
CXT 12	positive	0.157
5048	positive	1.695
5050	positive	0.490
CXT 10	positive	0.247
5053	positive	4.232
5055	positive	4.364
CXT 9	positive	2.455
5058	positive	3.886
5059	positive	4.450
CXT 8	positive	4.374
5061	positive	4.032
CXT 7	positive	0.647
CXT 6	positive	4.592

***H. pylori* ELISA (n = 1821)**

		***H. pylori* infection status**	
		positive	**negative**
H. pylori stool sandwich ELISA	**positive**	89	6
cut off $OD_{450-620}$: 0.09	**negative**	5	82

Sensitivity: 94.7%
Specificity: 93.2%

Table 4 shows the results of the analysis of *H. pylori*-negative and *H. pylori*-positive stool samples by means of stool sandwich ELISA. Here, the monoclonal antibody, preferably HP25.2m/2H10, is used both as catching and detection antibody (POD-labelled) for the detection of the *H. pylori* antigen catalase from the stool sample. The catalase is an extremely stable antigen which passes the digestive tract almost unchanged and can thus be detected in the stool sample. The analysis of 182 stool samples in the purely monoclonal ELISA system, which is based on only one catalase-specific mab, has a sensitivity of 94.7% and a specificity of 93.2%. This sensitivity and specificity leads to such high positive and negative predicative values that it is possible to detect an infection with H. pylori with sufficient reliability merely via the simple, inexpensive and non-invasive stool analysis in order to decide on an eradication treatment. Possibly, the sensitivity and specificity can be increased by a combination of different mabs which are directed against different epitopes of the catalase or by a combination of two detection systems for different antigens (e.g. catalase/urease).
Due to the development of a one-step ELISA test, improved applicability compared to the three-step ELISA test, described hereafter (Examples 11 and 12) was achieved.

Example 11: Finding suitable antibody pairings in a three-step ELISA

The test was carried out according to Example 10.
For finding suitable antibody pairings, the monoclonal antibodies against catalase (cf. Table 3), which had been purified and some of which had been biotinylated (cf.

Example 7), were at disposal. Firstly, the antibodies were titrated against each other to find the optimal concentration for use as catching and detector antibody. Then, patients' stool samples were tested using the ELISA systems that had been optimised in that way and the detection limits of catalase in human *H. pylori*-negative stool (zero-stool) was determined (Table 5).

Suitable mab combinations with regard to patient recognition and antigen detection-limit are shown in Table 5.

Table 5: Results of finding pairings of monoclonal antibodies against catalase (three-step ELISA)

	catching antibody				
biotinylated detection antibody	25.2m/ 2H10	25.6m/ 1B5	25.6m/ 1G4	25.6m/ 1A5	25.6m/ 1H4
25.2m/2H10	N: 0.03	0.1	0.03	0.1	0.03
	G4: 7-8	7	8	7	8
	G0: 2	2	2	2	2
25.6m/1B5	N: 0.1	0.1	0.1	0.03	0.3
	G4: 8	7	5	7	8
	G0: 2	1	2	2	2
25.6m/1G4	N: 0.3	0.1	0.1	0.01	0.1
	G4: 6-8	7	8	8	8
	G0: 1-2	2	4	2	2
25.6m/1A5	N: 0.3	0.1	0.3	0.1	0.3
	G4: 6-7	7	5	7	8
	G0: 2	2	2	2	2
25.6m/1H4	N: 0.1	0.3	0.1	0.3	0.1
	G4: 8		4-7	7	8
	G0: 3		2	2	2

patient recognition (detection of 8 critical-positive G4 and 4 clinical-negative G0 samples)
N = detection limit [ng/ml] of the catalase in zero-stool
critical-positive = samples which turned out to be particularly problematic in the detection

Finding suitable antibody pairings in a one-step ELISA

For finding suitable antibody pairings, monoclonal antibodies against catalase (cf. Table 9), which had been purified and some of which had been labelled with peroxidase (cf. Example 7), were at disposal.
The different combinations of catching and detection antibodies (cf. Table 6) were tested in a one-step ELISA using 27 *H. pylori*-positive and 17 *H. pylori*-negative patients' samples.

One-step sandwich ELISA

The ELISA plate (MaxiSorb Lockwell; Nunc) was coated over night at 2-8°C with 100 µl of an mab solution (2.0 µg catching antibodies/ml carbonate buffer, 0.1 M, pH 9.5). The ELISA plates coated in this way were washed twice with PBS and 200 µl blocking buffer (0.3% BSA; 20% sorbitol in PBS) per well were added and incubated over night at 2-8°C to block the binding sites that were still free. The ELISA plates were sucked off, dried over night in a circulating drying oven at 28°C and then stored with desiccant bags at 2-8°C.
Patients' stool was suspended in sample buffer (150 mM PBS + 0.5% animal serum + 1 mM EDTA + 0.1% detergent) at a ratio of 1:5 (0.1 g stool sample + 500 µl sample buffer) for approximately 30 sec (Vortex) and then centrifuged at 3000 g for 5 min. Per well, 50 µl of the supernatant were applied to the plate.
Subsequently, 50 µl of the POD-labelled antibody which had been diluted in sample buffer (0.5 nM ab-dextrane POD or 0.2 µg/ml HP25.2m/2H10-POD-P) were added directly to the stool suspension. The plates were incubated on a shaker for 1 hour.
After washing with washing buffer (75 mM PBS, 0.25% Tween) five times, the peroxidase substrate TMB (tetramethyl benzidine) was added to the one-component substrate (Neogen) (100 µl/well). After 10 min, the enzyme reaction was

stopped by adding 1 N hydrochloric acid (100 µl/well). Then, the intensity of the colouring was measured at 450 nm against the reference wave length of 630 nm.

Table 6: Results of the pairings of monoclonal antibodies against catalase (one-step ELISA)

	catching antibody								
POD-labelled detection antibody	25.2m/ 2H10	25.6m/ 1B5	25.6m/ 1A5	25.6m/ 4A12	25.6m/ 1G4	25.6m/ 1H4	25.6m/ 3D6	25.6m/ 2E12	25.6m/ 5E4
25.2m/2H10	-	24 14	24 11	22 13	23 14	24 12	19 14	24 12	22 15
25.6m/1B5	G4: 23 G0: 12	-	23 13	20 14	23 12	23 12	18 12	23 10	22 12
25.6m/1H4	G4: 21 G0: 9	24 13	24 14	-	24 11	-	20 15	25 12	24 20
25.6m/4A12	G4: 20 G0: 13	20 12	20 17	-	20 13	25 3	19 13	20 13	20 15
25.6m/3D6	G4: 17 G0: 15	24 9	24 12	21 13	23 8	23 13	-	22 9	22 14

patient recognition (detection of 27 critical-positive G4 and 17 clinical-negative G0 samples); cut off $OD_{450\text{-}630\ nm}$: 0.15.

The antibody combination HP25.6m/1B5 (catching antibody) and HP25.2m/2H10-POD (detection antibody) proved to be most advantageous due to the good patient recognition (correct detection of 24 out of 27 *H. pylori*-positive and 14 out of 17 *H. pylori*-negative samples), and the great signal intensity for detecting *H. pylori* antigens (catalase) in human stool.

Example 12: Detection of *H. pylori* in human stool by means of one-step ELISA

For the test, stool samples of patients from 10 different hospitals or gastroenterological surgeries were at disposal. The *H. pylori*-negative or *H. pylori*-

positive status was determined by means of ^{13}C urea breath test and/or histological analyses of gastric biopsies.

H. pylori stool sandwich ELISA (one-step test)

The ELISA plate (MaxiSorb Lockwell; Nunc) was coated over night at 2-8°C with 100 µl HP25.6m/1B5)/ml carbonate buffer, 0.1 M, pH 9.5). The ELISA plates coated in this way were washed twice with PBS. 200 µl blocking buffer (0.3% BSA; 20% sorbitol in PBS) per well were added and incubated over night at 2-8°C to block the binding sites that were still free. The ELISA plates were sucked off, dried over night in a circulating drying oven at 28°C and then stored with desiccant bags at 2-8°C.
Patients' stool was suspended in sample buffer (150 mM PBS + 0.5% animal serum + 1 mM EDTA + 0.1% detergent) at a ratio of 1:5 (0.1 g stool sample + 500 µl sample buffer) for approximately 30 sec (Vortex) and then centrifuged at 3000 g for 5 min. Per well, 50 µl of the supernatant (double to threefold determination) were applied to the plate. Subsequently, 50 µl of the POD-labelled antibody HP25.2m/2H10-dextrane POD, which had been diluted in sample buffer were added directly to the stool suspension. The plates were incubated on a shaker for 1 hour.
After washing with washing buffer (75 mM PBS, 0.25% Tween) five times, the peroxidase substrate TMB (tetramethyl benzidine) was added to the one-component substrate (Neogen) (100 µl/well). After 10 min, the enzyme reaction was stopped by adding 1 N hydrochloric acid (100 µl/well). Then, the intensity of the colouring was measured at 450 nm against the reference wave length of 630 nm.

Table 7: Comparison of the test results of the one-step ELISA test with the gold standard in the analysis of 199 stool samples altogether

number of the sample	result of the C13 breath test	result of the gastric biopsy	result of the one-step ELISA
1001	n.d.	negative	0.033
1002	n.d.	negative	0.022
1007	n.d.	negative	0.015
1008	n.d.	negative	0.032
1010	n.d.	negative	0.016

1012	n.d.	negative	0.026
1017	n.d.	negative	0.026
1021	n.d.	negative	0.014
1022	n.d.	negative	0.018
1024	n.d.	negative	0.018
1025	n.d.	negative	0.022
1027	n.d.	negative	0.044
1030	n.d.	negative	0.021
1031	n.d.	negative	0.014
1032	n.d.	negative	0.014
1034	n.d.	negative	0.023
1035	n.d.	negative	0.068
1040	n.d.	negative	0.058
1046	n.d.	negative	0.023
2002	n.d.	negative	0.019
2006	n.d.	negative	0.017
2007	negative	n.d.	0.019
2010	n.d.	negative	0.070
2012	negative	n.d.	0.040
2013	negative	n.d.	0.040
2014	negative	n.d.	0.016
2015	n.d.	negative	0.027
2017	negative	negative	0.034
2018	negative	negative	0.030
2023	n.d.	negative	0.031
2024	negative	n.d.	0.023
2028	n.d.	negative	0.049
2033	negative	negative	0.040
2034	negative	negative	0.083
2043	n.d.	negative	0.083
3123	negative	n.d.	0.013
3213	n.d.	negative	0.035
3224	negative	n.d.	0.014
3225	n.d.	negative	0.025
4004	n.d.	negative	0.044
5004	n.d.	negative	0.045
5007	n.d.	negative	0.014
5008	n.d.	negative	0.015
5009	n.d.	negative	0.028
5010	n.d.	negative	0.058
5012	n.d.	negative	0.030
5013	n.d.	negative	0.031
5017	n.d.	negative	0.027
5018	n.d.	negative	0.033
5019	n.d.	negative	0.010
5020	n.d.	negative	0.192
5021	n.d.	negative	0.023
5022	n.d.	negative	0.017
5024	n.d.	negative	0.011
5025	n.d.	negative	0.015
5027	n.d.	negative	0.026
5028	n.d.	negative	0.020
5030	n.d.	negative	0.033
5031	n.d.	negative	0.013
5033	n.d.	negative	0.014
5035	n.d.	negative	0.028
5036	n.d.	negative	0.022
5040	n.d.	negative	0.024
5042	n.d.	negative	0.053
5046	n.d.	negative	0.018
5052	n.d.	negative	0.015
5056	n.d.	negative	1.919

5057	n.d.	negative	0.015
5060	n.d.	negative	0.027
5063	n.d.	negative	0.010
5064	n.d.	negative	0.010
5066	n.d.	negative	0.020
5067	n.d.	negative	0.041
5068	n.d.	negative	0.017
6002	n.d.	negative	0.024
6005	n.d.	negative	0.023
6008	n.d.	negative	0.054
6009	n.d.	negative	0.065
6017	n.d.	negative	0.024
6024	n.d.	negative	0.050
6026	n.d.	negative	0.017
6029	n.d.	negative	0.014
6033	n.d.	negative	0.013
6038	n.d.	negative	0.019
6039	n.d.	negative	0.015
7005	n.d.	negative	0.031
7009	n.d.	negative	0.039
7013	n.d.	negative	0.026
8004	n.d.	negative	0.015
8047	n.d.	negative	0.042
9004	n.d.	negative	0.012
9005	n.d.	negative	0.105
9010	n.d.	negative	0.054
9011	n.d.	negative	0.647
9012	n.d.	negative	0.026
9013	n.d.	negative	0.022
9015	n.d.	negative	0.032
9019	n.d.	negative	0.040

9022	n.d.	negative	0.029
213	n.d.	positive	0.752
444	n.d.	positive	0.241
1003	n.d.	positive	0.446
1013	n.d.	positive	3.809
1014	n.d.	positive	0.316
1015	n.d.	positive	2.693
1028	n.d.	positive	0.959
1029	n.d.	positive	4.336
1037	n.d.	positive	2.152
2005	positive	n.d.	1.289
2008	n.d.	positive	3.814
2009	positive	n.d.	1.050
2016	n.d.	positive	1.564
2029	positive	positive	4.347
2032	positive	positive	2.661
2035	n.d.	positive	3.632
2039	positive	positive	0.694
2040	n.d.	positive	3.189
2041	positive	positive	1.195
2042	positive	positive	4.350
3146	positive	n.d.	4.189
3219	positive	positive	4.267
3220	positive	positive	4.138
3231	positive	positive	4.332
3234	positive	positive	3.989
3241	positive	positive	1.580
3570	positive	n.d.	4.147
4003	n.d.	positive	4.140
4005	positive	positive	0.298
4006	n.d.	positive	4.228

4018	n.d.	positive	3.319
4019	n.d.	positive	2.892
4020	n.d.	positive	1.167
5001	n.d.	positive	4.438
5006	n.d.	positive	4.343
5029	n.d.	positive	1.354
5039	n.d.	positive	4.401
5048	n.d.	positive	2.805
5050	n.d.	positive	0.744
5053	n.d.	positive	3.896
5055	n.d.	positive	3.825
5058	n.d.	positive	4.153
5061	n.d.	positive	4.050
5069	n.d.	positive	1.411
5072	n.d.	positive	4.322
5075	n.d.	positive	4.285
5076	n.d.	positive	4.402
5078	n.d.	positive	1.319
5090	n.d.	positive	4.268
5092	n.d.	positive	1.975
5100	n.d.	positive	2.406
5150	n.d.	positive	0.132
6001	n.d.	positive	4.325
6004	n.d.	positive	4.035
6013	n.d.	positive	2.684
6014	n.d.	positive	4.209
6015	n.d.	positive	4.164
6018	n.d.	positive	4.551
6020	n.d.	positive	0.376
6022	n.d.	positive	1.915
6027	n.d.	positive	4.244

6040	n.d.	positive	3.105
6050	n.d.	positive	3.806
6052	n.d.	positive	4.221
6064	n.d.	positive	4.225
6065	n.d.	positive	4.210
7001	n.d.	positive	2.584
7002	n.d.	positive	4.245
7003	n.d.	positive	2.236
7020	n.d.	positive	0.038
8026	n.d.	positive	0.013
8033	n.d.	positive	1.269
9001	n.d.	positive	3.765
9002	n.d.	positive	4.049
9003	n.d.	positive	3.674
9006	n.d.	positive	0.992
9007	n.d.	positive	0.052
9008	n.d.	positive	4.165
9009	n.d.	positive	0.033
9014	n.d.	positive	4.042
9017	n.d.	positive	4.276
9018	n.d.	positive	0,44
9022	n.d.	positive	1.961
T 01	positive	n.d.	2.083
T 02	positive	n.d.	1.722
T 03	positive	positive	3.871
T 04	positive	positive	4.463
T 05	positive	positive	2.368
T 07	positive	positive	0.785
T 09	positive	n.d.	1.480
T 10	positive	n.d.	0.768
T 13	n.d.	positive	2.211

T 53	positive	n.d.	4.500
T 58	positive	n.d.	1.540
T 64	positive	n.d.	1.879
T 67	positive	n.d.	1.608
T 68	positive	n.d.	4.377

T 70	positive	n.d.	0.675
T 77	positive	n.d.	0.038
T 88	positive	n.d.	1.377

n.d.: not determined

cut off: (OD 450-630nm): positive ≥ 0.18; negative ≤ 0.13

(n=199)

method		**gold standard**	
		positive	**negative**
one-step test	**positive**	**94**	**3**
	negative	**6**	**96**

Sensitivity: 94%

Secificity: 97%

Result:

Table 7 shows the results of the examination of *H. pylori*-negative and *H. pylori*-positive stool samples using a one-step stool sandwich ELISA. The monoclonal antibodies (HP25.6m/1B5; HP25.2m/2H10) were used for detecting the *H. pylori* antigen catalase from stool samples. The examination of 199 stool samples in the purely monoclonal ELISA system, which is based on the aforementioned catalase-specific mab, has a sensitivity of 94% and a specificity of 97%.

Example 13: Detection of *H. pylori* in human stool by means of an optimised one-step ELISA

For the test, 357 stool samples of patients from 10 different hospitals or gastroenterological surgeries were at disposal. The *H. pylori* status was determined by means of histological analyses of gastric biopsies. The tests were carried out in an external GLP laboratory with the test samples being coded so that the laboratory staff did not know the status of the infection of the samples.

Optimised one-step sandwich ELISA:

The ELISA plate (MaxiSorb Lockwell; Nunc) was coated over night at 2-8°C with 100 µl of an mab solution (2.0 µg of HP25.6m/1B5)/ml carbonate buffer, 0.1 M, pH 9.5). The ELISA plates coated in this way were washed twice with PBS. 200 µl blocking buffer (0.3% BSA; 5% sorbitol in PBS) per well were added and incubated over night at 2-8°C to block the binding sites that were still free. The ELISA plates were sucked off, dried over night in a circulating drying oven at 28°C and then stored with desiccant bags at 2-8°C.
Patients' stool was suspended in sample buffer (150 mM PBS + 0.5% animal serum + 1 mM EDTA + 0.1% detergent) at a ratio of 1:5 (0.1 g stool sample + 500 µl sample buffer) for approximately 30 sec (Vortex) and then centrifuged at 3000 g for 5 min. Per well, 50 µl of the supernatant were applied to the plate. Subsequently, 50 µl of the POD-labelled antibody (200 femtomol/ml HP25.2m/2H10-dextrane POD-labelled), which had been diluted in sample buffer, were added directly to the stool suspension. The plates were incubated on a shaker for 1 hour. After washing with washing buffer (75 mM PBS, 0.25% Tween) five times, the peroxidase substrate TMB (tetramethyl benzidine) was added to the one-component substrate (Seramun) (100 µl/well). After 10 min, the enzyme reaction was stopped by adding 1 M sulfuric acid (100 µl/well). Then, the intensity of the colouring was measured at 450 nm against the reference wave length of 630 nm.

Table 8: *H. pylori* stool sandwich ELISA (optimised one-step test)

Detection of *H. pylori* catalase in stool by means of an optimised one-step ELISA using the monoclonal antibodies HP25.6m/1B5; HP25.2m/2H10.

patient no.	histological result	HP stool ELISA
CX 7042	negative	0.022
CX 12070	negative	0.018
CX 9138	negative	0.013
CX 12080	negative	0.015
CX 12076	negative	0.071
CX 7028	negative	0.019
CX 9046	negative	0.013
CX 12077	negative	0.025
CX 9109	negative	0.012
CX 9120	negative	0.018
CX 9144	negative	0.014
CX 12032	negative	0.017
CX 2067	negative	0.037
CX 8010	negative	0.017
CX 12027	negative	0.043
CX 12085	negative	0.012
CX 2105	negative	0.016
CX 9029	negative	0.028
CX 9101	negative	0.013
CX 9119	negative	0.073
CX 9129	negative	0.022
CX 9174	negative	0.029
CX 12079	negative	0.016
CX 12092	negative	0.031
CX 2066	negative	0.043
CX 5115	negative	0.022
CX 7035	negative	0.076
CX 9024	negative	0.018
CX 9136	negative	0.014
CX 12065	negative	0.017
CX 12084	negative	0.014
CX 2044	negative	0.028
CX 7032	negative	0.048
CX 8011	negative	0.014
CX 8050	negative	0.015
CX 9056	negative	0.014
CX 6067	negative	0.016
CX 9041	negative	0.036
CX 9157	negative	0.021
CX 12042	negative	0.014
CX 9134	negative	0.016
CX 9160	negative	0.015
CX 9171	negative	0.042
CX 9025	negative	0.017
CX 9150	negative	0.014
CX 2050	negative	0.013
CX 2057	negative	0.021
CX 9184	negative	0.018
CX 11021	negative	0.009

CX 7043	negative	0.024
CX 7036	negative	0.016
CX 7047	negative	0.015
CX 9064	negative	0.06
CX 8002	negative	0.015
CX 9115	negative	0.016
CX 9189	negative	0.063
CX 9195	negative	0.015
CX 12059	negative	0.028
CX 8015	negative	0.015
CX 9137	negative	0.052
CX 9187	negative	0.015
CX 9047	negative	0.017
CX 9166	negative	0.019
CX 12064	negative	0.031
CX 2070	negative	0.018
CX 6081	negative	0.05
CX 9104	negative	0.013
CX 9167	negative	0.017
CX 9196	negative	0.027
CX 9066	negative	0.016
CX 10010	negative	0.012
CX 9061	negative	0.014
CX 9170	negative	0.013
CX 11012	negative	0.03
CX 2064	negative	0.024
CX 5101	negative	0.025
CX 7021	negative	0.045
CX 9105	negative	0.013
CX 12016	negative	0.019
CX 6070	negative	0.013

CX 2101	negative	0.021
CX 8014	negative	0.016
CX 9169	negative	0.014
CX 12088	negative	0.017
CX 9121	negative	0.033
CX 9023	negative	0.055
CX 12071	negative	0.022
CX 10003	negative	0.028
CX 12047	negative	0.02
CX 9089	negative	0.017
CX 9107	negative	0.032
CX 2061	negative	0.03
CX 11013	negative	0.014
CX 9092	negative	0.017
CX 12021	negative	0.049
CX 12024	negative	0.023
CX 9125	negative	0.019
CX 2107	negative	0.025
CX 9039	negative	0.032
CX 12046	negative	0.013
CX 11024	negative	0.053
CX 12012	negative	0.015
CX 12040	negative	0.028
CX 2087	negative	0.027
CX 9028	negative	0.018
CX 9176	negative	0.014
CX 10007	negative	0.019
CX 12089	negative	0.012
CX 7048	negative	0.041
CX 9114	negative	0.019
CX 12019	negative	0.028

CX 7033	negative	0.081
CX 9067	negative	0.016
Cx 9108	negative	0.165
CX 9197	negative	0.016
CX 5133	negative	0.219
CX 9094	negative	0.041
CX 10021	negative	0.019
CX 12090	negative	0.012
CX 9116	negative	0.018
CX 10037	negative	0.019
CX 12049	negative	0.016
CX 12093	negative	0.026
CX 9097	negative	0.02
CX 9183	negative	0.025
CX 11023	negative	0.068
CX 5114	negative	0.061
CX 9161	negative	0.017
CX 2068	negative	0.027
CX 8005	negative	0.025
CX 9179	negative	0.015
CX 12001	negative	0.028
CX 9062	negative	0.022
CX 9118	negative	0.013
CX 6071	negative	0.027
CX 9035	negative	0.016
CX 10006	negative	0.017
CX 9095	negative	0.018
CX 9199	negative	0.016
CX 10018	negative	0.019
CX 12008	negative	0.018
CX 9052	negative	0.015

CX 9181	negative	0.014
CX 12058	negative	0.055
CX 9030	negative	0.023
CX 9059	negative	0.015
CX 10005	negative	0.028
CX 10039	negative	0.018
CX 9190	negative	0.015
CX 9164	negative	0.016
CX 10044	negative	0.023
CX 9110	negative	0.027
CX 9127	negative	0.018
CX 12013	negative	0.022
CX 5105	negative	0.017
CX 9178	negative	0.037
CX 10024	negative	0.015
CX 2098	negative	0.038
CX 10008	negative	0.015
CX 10034	negative	0.016
CX 9162	negative	0.513
CX 12023	negative	0.023
CX 2091	negative	0.225
CX 12034	negative	0.022
CX 12039	negative	0.019
CX 9085	negative	0.022
CX 10029	negative	0.03
CX 11022	negative	0.031
CX 2073	negative	0.035
CX 12017	negative	0.017
CX 9141	negative	0.024
CX 10026	negative	0.014
CX 12003	negative	0.038

CX 7049	negative	0.028
CX 9026	negative	0.026
CX 10011	negative	0.012
CX 9124	negative	0.02
CX 12015	negative	0.029
CX 10022	negative	0.021
CX 10001	negative	0.017
CX 7040	negative	0.014
CX 9048	negative	0.017
CX 6075	negative	0.024
CX 10016	negative	0.024
CX 9073	negative	0.015
CX 9081	negative	0.036
CX 12007	negative	0.034
CX 9122	negative	0.078
CX 9069	negative	0.025
CX 9091	negative	0.029
CX 10012	negative	0.034
CX 10027	negative	0.07
CX 10009	negative	0.023
CX 10014	negative	0.021
CX 9040	negative	0.038
CX 9090	negative	0.027
CX 12006	negative	0.026
CX 9060	negative	0.013
CX 10031	negative	0.023
CX 9075	negative	0.019
CX 5131	negative	0.032
CX 9054	negative	0.016
CX 9070	negative	0.022
CX 12099	negative	0.014

CX 9050	negative	0.038
CX 9086	negative	0.017
CX 10013	negative	0.036
CX 12062	negative	4
CX 6063	negative	3.537
CX 9133	positive	0.023
CX 9188	positive	0.017
CX 9192	positive	0.014
CX 2048	positive	0.548
CX 2078	positive	0.296
CX 8009	positive	0.695
CX 9145	positive	1.778
CX 9076	positive	0.09
CX 9072	positive	0.024
CX 5148	positive	0.213
CX 11004	positive	0.477
CX 2093	positive	0.271
CX 12060	positive	1.205
CX 7053	positive	2.436
CX 11006	positive	0.13
CX 8001	positive	4
CX 2100	positive	1.539
CX 5113	positive	0.583
CX 7029	positive	0.155
CX 10020	positive	1.335
CX 2099	positive	3.403
CX 12018	positive	0.927
CX 7037	positive	4
CX 2083	positive	3.896
CX 4001	positive	0.678
CX 5125	positive	4

CX 9049	positive	0.588
CX 5112	positive	1.797
CX 9142	positive	3.122
CX 7044	positive	2.155
CX 2109	positive	3.786
CX 8012	positive	4
CX 4011	positive	3.376
CX 10049	positive	2.98
CX 5128	positive	3.348
CX 10038	positive	3.652
CX 12067	positive	2.928
CX 4029	positive	3.087
CX 2104	positive	2.855
CX 11003	positive	0.786
CX 9065	positive	1.324
CX 12048	positive	2.409
CX 12051	positive	4
CX 10015	positive	4
CX 7024	positive	4
CX 12091	positive	4
CX 5126	positive	3.834
CX 7057	positive	4
CX 5120	positive	1.935
CX 11002	positive	0.378
CX 11011	positive	4
CX 2102	positive	2.452
CX 2103	positive	3.091
CX 11010	positive	1.905
CX 5108	positive	3.58
CX 9130	positive	2.499
CX 11008	positive	3.367

CX 9194	positive	4
CX 12028	positive	3.671
CX 4016	positive	2.545
CX 4013	positive	4
CX 9135	positive	4
CX 11001	positive	4
CX 2106	positive	2.71
CX 2094	positive	4
CX 9082	positive	1.769
CX 5123	positive	3.773
CX 6076	positive	4
CX 9155	positive	4
CX 7030	positive	3.661
CX 9128	positive	4
CX 12035	positive	4
CX 10023	positive	3.426
CX 2060	positive	4
CX 12041	positive	4
CX 9045	positive	1.382
CX 9096	positive	1.653
CX 2056	positive	4
CX 12002	positive	2.441
CX 6061	positive	0.018
CX 11020	positive	4
CX 9147	positive	3.758
CX 9078	positive	3.686
CX 5147	positive	4
CX 7023	positive	4
CX 9131	positive	4
CX 9156	positive	4
CX 10019	positive	3.438

CX 12026	positive	3.941
CX 9079	positive	3.628
CX 4023	positive	4
CX 9031	positive	3.273
CX 5116	positive	4
CX 9077	positive	3.929
CX 4012	positive	4
CX 5106	positive	3.648
CX 12095	positive	4
CX 10002	positive	3.698
CX 11005	positive	4
CX 9093	positive	4
CX 11014	positive	3.465
CX 9051	positive	4
CX 10028	positive	3.799
CX 4017	positive	4
CX 9182	positive	4
CX 9099	positive	4
CX 12022	positive	4
CX 2079	positive	3.884
CX 9102	positive	3.524
CX 2076	positive	3.593
Cx 9177	positive	4
CX 9088	positive	2.14
CX 6072	positive	4
CX 7038	positive	4
CX 9123	positive	4
CX 12074	positive	4
CX 9055	positive	4
CX 9036	positive	4
CX 6078	positive	4

CX 2069	positive	3.778
CX 9043	positive	3.727
CX 12050	positive	3.516
CX 5119	positive	4
CX 9113	positive	4
CX 9068	positive	3.857
CX 2092	positive	4
CX 10050	positive	4
CX 9053	positive	3.874
CX 4015	positive	3.784
CX 12075	positive	3.717
CX 9027	positive	3.718
CX 9080	positive	4
CX 9098	positive	4
CX 9112	positive	4
CX 9175	positive	4
CX 9063	positive	4
CX 12020	positive	4
CX 9158	positive	4
CX 9198	positive	3.874
CX 9165	positive	4
CX 9034	positive	3.874
CX 12055	positive	3.754
CX 6074	positive	4
CX 6082	positive	4
CX 6069	positive	4
CX 9193	positive	4
CX 9149	positive	4
CX 9106	positive	4

Cut off: OD $_{450\text{-}640nm}$: 0.15

n= 357

		histology	
		positive	negative
HP stool ELISA	positive	141	6
	negative	7	203

Sensitivity: 95 % **confidence interval (95%): 90.5 – 98.1 %**
Specificity: 97 % **confidence interval (95%): 93.9 – 98.0 %**

Result:

Table 8 shows the results of the examination of *H. pylori*-negative and *H. pylori*-positive stool samples (first diagnosis) by means of a stool sandwich ELISA. Monoclonal antibodies (catching antibody: HP25.6m/1B5; detection antibody HP25.2m/2H10-POD) were used for detection of the *H. pylori* antigen catalase in the stool sample. The analysis of stool samples in a purely monoclonal ELISA system, which is based on catalase-specific mabs, has a sensitivity of 95% and a specificity of 97%.

Example 14: Course of eradication/eradication control

An eradication control can only be carried out via a direct detection of *H. pylori* antigens and not of antigens in serum since, after an infection, *H. pylori* antibodies are still present in the blood for many months. Thus, in contrast to serologic *H. pylori* tests, the described sandwich stool ELISA offers the possibility of assessing the success of an eradication. Fig. 9 shows the course of an eradication treatment of a *H. pylori*-positive patient after application of Omeprazol, Metronidazol and Clarithromycin. 6 days after beginning of the therapy, no *H. pylori* antigen could be detected in the stool any more.

Table 9 shows the results of the HP stool ELISA in an eradication study. 4 to 6 weeks after the eradication therapy, the stool samples were taken. The ^{13}C-breath test served as a reference test.
The tests were carried out according to Example 12 (one-step ELISA).

Table 9: Eradication control – detection of *H. pylori* – catalse from the stool by means of one-step ELISA. Taking of the samples: 4-6 weeks after eradication therapy.

patient no.	^{13}C-breath test	HP stool ELISA OD 450-630nm
131	negative	0.024
132	negative	0.012
138	negative	0.024
147	negative	0.016
148	negative	0.014
149	negative	0.019
151	negative	0.018
154	negative	0.012
155	negative	0.011
158	negative	0.013
159	negative	0.023
161	negative	0.025
165	negative	0.013
166	negative	0.014
167	negative	0.183
168	negative	0.016
172	negative	0.015
177	negative	0.015
180	negative	0.146
182	negative	0.026
187	negative	0.014
188	negative	0.017
194	negative	0.020
195	negative	0.015
199	negative	0.013
205	negative	0.035
206	negative	0.020
213	negative	0.018
215	negative	0.014
216	negative	0.034
217	negative	0.014
219	negative	0.014
223	negative	0.086
224	negative	0.020
227	negative	0.139
245	negative	0.094
246	negative	0.120

250	negative	0.019
251	negative	0.042
253	negative	0.034
255	negative	0.033
256	negative	0.041
270	negative	0.053
271	negative	0.033
275	negative	0.040
283	negative	0.036
284	negative	0.018
296	negative	0.170
303	negative	0.064
308	negative	0.029
310	negative	0.025
311	negative	0.013
312	negative	0.049
315	negative	0.021
318	negative	0.037
319	negative	0.044
320	negative	0.057
322	negative	0.019
324	negative	0.017
326	negative	0.154
327	negative	0.016
328	negative	0.015
329	negative	0.266
330	negative	0.035
331	negative	0.013
337	negative	0.015
338	negative	0.051
339	negative	0.021
350	negative	0.037
353	negative	0.019
356	negative	0.023
357	negative	0.025
358	negative	0.057
359	negative	0.023
360	negative	0.073
366	negative	0.018
367	negative	0.018
368	negative	0.029
369	negative	0.028
152	positive	0.365
156	positive	0.264
160	positive	3.851
162	positive	2.021
169	positive	0.112
179	positive	0.573
181	positive	2.886
186	positive	2.084
196	positive	0.282
220	positive	0.905
240	positive	2.837
252	positive	1.606
278	positive	3.173
300	positive	0.840
325	positive	3.898
334	positive	2.946
361	positive	0.269
161/ 1799	positive	0.263

In comparison to the reference test, the study (97 patients) shows a sensitivity of 94% and a specificity of 95% (cut off: $OD_{450-630}$: 0.15).

Example 14 shows that HP stool ELISA cannot only be used for a first diagnosis of *H. pylori*, but also for controlling eradication and documenting the course of eradication.

Example 15: Cloning and sequence determination of the functional variable regions of immunoglobulins from hybridoma cell lines

Total RNA was isolated from antibody-producing hybridoma cell lines according to Chomczynski (Chomczynski, 1987).
Then, the corresponding cDNA was synthesized according to standard methods (Sambrook et al., 1989).
The DNA region encoding the kappa light chain as well as the heavy chain Fd segment (VH or CH1) of the respective antibodies were amplified by means of PCR. The oligonucleotide primer set stated in Table 10 was used, the cDNA isolated from the single hybridoma cell lines served as a template.
The primer set used leads to a 5'-*XhoI* and a 3'-*Spe*I cleavage site in the heavy chain Fd fragments as well as to a 5'-*Sac*I and a 3'-*Xba*I cleavage site in the kappa light chains. For PCR amplification of the DNA fragments encoding the heavy chain Fd, 11 different 5'-VH primers (MVH 1-8 and MULH 1-3) were each combined with the 3'-VH primer MIgG2a [HP25.2m/2H10] or used with 3'-VH primer MIgG1 [HP25.6m/1B5]. For the amplification of the DNA fragments which encode the kappa light chains, 11 different 5'-VK primers (MUVK 1-7 and MULK 1-4) were each combined with the 3'-VK primer 3'MUCK.
The following temperature program was used in all PCR amplifications: denaturation at 94°C for 30 s, primer attachment at 52°C for 60 s, polymerization at 72°C for 90 s. This program was maintained for 40 cycles, followed by a final completion of the fragments at 72°C for 10 min.
The results of the PCR amplifications were separated by means of agarose gel electrophoresis and the DNA bands of the expected molecular weight were isolated. For the antibody (HP25.2m/2H10), the isolated bands were subjected to a

restriction digestion using the enzymes XhoI and SpeI (heavy chains) or SacI and XbaI (light chains). The fragments obtained were cloned into the plasmid vector Bluescript KS (Stratagene) after the vector had first been cleaved with the restriction enzymes XhoI and SpeI or SacI and XbaI.
Subsequently, plasmid preparations of the cloned heavy and light chain fragments were sequenced. Sequences were chosen which encode the functional variable regions of the heavy and light chains of immunoglobulin (VH or VL). In this way, it was possible to identify exactly one functional VH and one functional VL region for each hybridoma cell line. Fig. 1 and Fig. 2 show the functional VH and VL sequences. The first four amino acids of the VH region were completed by recloning. Cloning and sequencing were carried out according to standard methods (Sambrook et al., 1989).

For the antibody HP25.6m/1B5, the isolated bands were then sequenced directly and a functional light and a functional heavy chain were identified. The heavy-chain Fd fragment and the light chain were subsequently subjected to a restriction digestion using the enzymes *Xho*I and *Spe*I (heavy chain) or *Sca*I and *Xba*I (light chain) and the fragments obtained were cloned into the plasmid vector pBIIIHisEx (Connex) after said vector had been cleaved with the restriction enzymes *Xho*I and *Spe*I and *Sac*I and *Xba*I, respectively. Then, they were sequenced again.

In this way, exactly one functional VH and one functional VL region could be identified for this hybridoma cell line. The functional VH and VL sequences are shown in Fig. 3 and Fig. 4. For the VH and VL sequences, the mature N terminals are depicted as have been determined by sequencing via leader primers. Cloning and sequencing was carried out according to standard methods (Sambrook et al., 1989).

Table 10: List of the primers used for the PCR amplification of the functional variable regions of heavy and light immunoglobulin-chains (orientation 5' – 3')

MVH1	(GC)AG GTG CAG CTC GAG GAG TCA GGA CCT
MVH2	GAG GTC CAG CTC GAG CAG TCT GGA CCT
MVH3	CAG GTC CAA CTC GAG CAG CCT GGG GCT
MVH4	GAG GTT CAG CTC GAG CAG TCT GGG GCA
MVH5	GA(AG) GTG AAG CTC GAG GAG TCT GGA GGA
MVH6	GAG GTG AAG CTT CTC GAG TCT GGA GGT
MVH7	GAA GTG AAG CTC GAG GAG TCT GGG GGA
MVH8	GAG GTT CAG CTC GAG CAG TCT GGA GCT
MULK1	GGG GAG CTC CAC CAT GGA GAC AGA CAC ACT CCT GCT AT
MULK2	GGG GAG CTC CAC CAT GGA TTT TCA AGT GCA GAT TTT CAG
MULK3	GGG GAG CTC CAC CAT GGA GWC ACA KWC TCA GGT CTT TRT A
MULK4	GGG GAG CTC CAC CAT GKC CCC WRC TCA GYT YCT KGT
MIgG1	TAT GCA ACT AGT ACA ACC ACA ATC CCT GGG
MIgG2a	GAG AGA GGG GTT CTG ACT AGT GGG CAC TCT GGG CTC
MUVK1	CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT
MUVK2	CCA GTT CCG AGC TCG TGT TGA CGC AGC CGC CC
MUVK3	CCA GTT CCG AGC TCG TGC TCA CCC AGT CTC CA
MUVK4	CCA GTT CCG AGC TCC AGA TGA CCC AGT CTC CA
MUVK5	CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA
MUVK6	CCA GAT GTG AGC TCG TCA TGA CCC AGT CTC CA
MUVK7	CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA
MULH1	GGG CTC GAG CAC CAT GGR ATG SAG CTG KGT MAT SCT CTT
MULH2	GGG CTC GAG CAC CAT GRA CTT CGG GYT GAG CTK GGT TTT
MULH3	GGG CTC GAG CAC CAT GGC TGT CTT GGG GCT GCT CTT CT
3`MUCK	GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A

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